UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

AUTOIMUNIDADE E CÉLULAS REGULADORAS

T CD4^{+}CD25^{HIGH} NA IMUNODEFICIÊNCIA

COMUM VARIÁVEL

Susana Clara Barão Lopes da Silva dos Anjos

MESTRADO EM IMUNOLOGIA MÉDICA

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Good research brings you more questions than answers.

Sir John Vane, Nobel Prize 1982

RESUMO

Introdução: Vários mecanismos têm sido sugeridos para explicar a elevada prevalência de doenças autoimunes (DAIs) na Imunodeficiência Comum Variável (ICV). Procurámos avaliar a prevalência de DAIs numa população com IDCV, caracterizar estes doentes e verificar se um defeito quantitativo na população T $CD4^{+}CD25^{high}$ poderia estar associado à maior prevalência de autoimunidade na ICV.

Métodos: Foram incluídos 47 doentes com ICV sob terapêutica substitutiva com imunoglobulina endovenosa (IGEV). Através de revisão dos processos clínicos e entrevista individual foram recolhidos dados clínicos e laboratoriais relativamente às manifestações de apresentação e evolução clínica, incluindo DAIs e níveis séricos de imunoglobulinas no diagnóstico de ICV. Em estudo transversal, foi quantificada IgG sérica e populações T, B e NK e células T CD4CD25 por citometria de fluxo em sangue total.

Resultados: Foram diagnosticadas DAIs em 19 doentes (40,4%), sendo as citopénias autoimunes as mais frequentes. As DAIs foram diagnosticadas antes da ICV em 8 doentes, nenhum deles sob terapêutica imunossupressora no ano anterior ao diagnóstico de ICV. A idade média dos doentes com DAI era superior no momento do estudo, diagnóstico de ICV e no início da terapêutica com IGEV. Também apresentavam uma prevalência mais elevada de diarreia crónica não infecciosa e hiperplasia linfoide e IgG sérica mais elevada no diagnóstico. O estudo transversal não evidenciou diferenças significativas na IgG sérica pré-infusional ou populações linfocitárias entre doentes com e sem DAI. As frequências de $CD4^{+}CD25^{high}$ foram significativamente mais baixas em doentes com DAI comparados com doentes sem DAI e com controlos saudáveis e no conjunto dos doentes com ICV comparados com estes controlos.

Conclusões: Estes resultados sugerem que a deficiência quantitativa de $CD4^{+}CD25^{high}$ poderá contribuir para a elevada prevalência de DAIs na ICV. Uma avaliação longitudinal e mais detalhada da população T $CD4^{+}CD25^{high}$, incluindo marcadores fenotípicos adicionais e estudo funcional, contribuirão para clarificar esta questão.

PALAVRAS CHAVE

Imunodeficiência Comum Variável, autoimunidade; células imunoreguladoras, $CD4^{+}CD25^{high}$

ABSTRACT

Background: Several mechanisms have been proposed to explain the high incidence of autoimmune diseases (AID) in Common Variable Immunodeficiency (CVID). We aimed to evaluate AID frequency within a CVID population and to characterize patients with AID. We also investigated whether a quantitative defect in the immunoregulatory population $CD4^{+}CD25^{high}$ could be associated with increased prevalence of autoimmunity in CVID.

Methods: 47 patients with CVID on regular intravenous immunoglobulin substitution therapy were enrolled. Chart review and questionnaire-guided interview were used to collect clinical and laboratory data concerning presentation symptoms and clinical evolution, including AID. Serum immunoglobulins were quantified at diagnosis. A cross-sectional evaluation was performed before IVIG infusion, including serum IgG level, T, B and NK cell quantification by flow-cytometry in freshly whole blood. $CD4^{+}CD25^{+}$ cells were simultaneously quantified in whole blood by flow-cytometry and compared with age-matched healthy volunteers.

Results: AIDs were diagnosed in 19 patients (40.4%) and autoimmune cytopenias were the most frequent. AID was diagnosed before CVID in eight patients, none on immunosuppressors in the year before CVID diagnosis. Patients with AID were older at the time of the present evaluation, at CVID diagnosis and at beginning of IVIG. They also exhibited higher prevalence of chronic non-infectious diarrhea and lymphoid hyperplasia and higher serum IgG at diagnosis. There were no significant differences in IgG pre-infusional levels and lymphocyte subpopulations between patients with and without AID. $CD4^{+}CD25^{high}$ frequencies were significantly lower in patients with AID compared to those without AID and controls and in the whole group of CVID compared to controls.

Conclusions: Our results suggest that $CD4^{+}CD25^{high}$ deficiency may possibly contribute to the high incidence of AID in CVID. More detailed and longitudinal evaluation of $CD4^{+}CD25^{high}$ T cells in larger cohorts, including the use of additional markers and suppressor cells function assessment, will help to clarify this issue.

KEY-WORDS

Common variable immunodeficiency, autoimmune diseases, regulatory cells, $CD4^{+}CD25^{high}$

ÍNDICE

PREÂMBULO

A decisão de fazer o Mestrado em Imunologia Médica foi motivada essencialmente pela vontade de aprofundar o conhecimento na especialidade que escolhi para a prática clínica. Sendo parte integrante da formação e actividade em Imunoalergologia, as Imunodeficiências Primárias (IDPs) são, desde logo por motivos epidemiológicos, uma área com a qual contactamos com menor frequência, apesar de a prevalência global destas doenças se situar em 1: 20 000 nascimentos, se englobarmos todos os grupos de IDPs. A Imunodeficiência Comum Variável é a IDP sintomática mais frequente, encontrando-se actualmente em seguimento no Serviço de Imunoalergologia do HSM cerca de 35 doentes com ICV. São frequentemente casos complexos e absorventes, que se caracterizam por uma diversidade de patologias, com espectro de gravidade alargado.

Do ponto de vista conceptual, as IDPs são extremamente instrutivas, constituindo verdadeiros modelos vivos que nos permitem compreender melhor o sistema imunitário. Para além do desafio que a sua complexidade de diagnóstico e as dificuldades na evolução clínica e terapêutica oferecem, o seguimento de doentes com IDPs torna-se hoje cada vez mais gratificante. O investimento da investigação nesta área tem contribuído para uma significativa melhoria da qualidade de vida dos doentes.

Todos estes motivos em conjunto contribuíram para aumentar o meu interesse pelas IDPs e reforçam a pertinência da escolha deste tema, em particular da Imunodeficiência Comum Variável, para área de trabalho prático no Mestrado em Imunologia Médica.

No decurso do Internato Complementar, estagiei durante o primeiro trimestre de 2004 no Hospital Vall d`Hebron, em Barcelona, centro de referência para crianças e adultos com IDPs da Catalunha. O dinamismo da orientação da Dra Teresa Español e a excelente recepção por parte dos colegas espanhóis permitiram rentabilizar de forma extraordinária aqueles 3 meses.

No Hospital Vall d´Hebron encontram-se em seguimento cerca de 15 crianças e 70 adultos com ICV, a maioria dos quais recorre àquela instituição para terapêutica substitutiva com IGEV. A possibilidade de acesso a uma população alargada e a disponibilidade do Laboratório de Imunologia, permitiram realizar o presente trabalho. Tendo colocado a hipótese original de um defeito quantitativo das células reguladoras $CD4^{+}CD25^{high}$ poder estar associado à elevada incidência de doenças autoimunes na ICV, sublinho e agradeço a disponibilidade e a coragem de toda a equipa para colaborar sem reservas com esta ideia.

A realização do trabalho prático durante o tempo do estágio foi um objectivo extremamente ambicioso / exigente para este período, que no entanto contribuiu de forma decisiva para o seu sucesso. A avaliação clínica, baseada na revisão dos processos e entrevista clínica, foi obviamente afectada pelos condicionalismos da metodologia retrospectiva. O protocolo laboratorial foi desenhado em conjunto com a equipa do Laboratório de Imunologia, o qual suportou todos os encargos financeiros e num período *record* o integrou na sua rotina e nos demais trabalhos em curso.

Obtiveram-se resultados significativos relativamente à hipótese colocada, original e integrada em linhas actuais de investigação em autoimunidade e imunodeficiências primárias humorais. Estes resultados, aliados ao facto de a autoimunidade constituir um problema simultaneamente frequente e intrigante para aqueles que na prática clínica e no laboratório lidam com doentes com ICV, motivaram a elaboração em inglês do artigo ***$CD4^{+}CD25^{high}$ and Autoimmunity in Common Variable Immunodeficiency: searching new answers for an old question.***

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LISTA DE ABREVIATURAS

AID	*Autoimmune disease*
AIHA	*Autoimmune hemolytic anemia*
AZT	*Azathioprine*
BAFF-R	*B-cell activating factor receptor (BAFF-R)*
CD	*Crohn´s disease*
CT	*Computed tomography*
CTLA-4	*Cytotoxic T Lymphocyte associated-Antigen 4*
CVID	*Common Variable Immunodeficiency*
Cy	*Cyclosporine*
DAI	Doença autoimune
EDTA	*EthyleneDiamineTetrAcetic acid*
ELISA	*Enzyme-Linked Immunosorbent Assay*
ESID	*European Society for Primary Immunodeficiencies*
F	*Female*
FITC	*Fluorescein isothiocyanate*
FoxP3	*Forkhead transcription factor*
GITR	*Glucocorticoid-Induced TNF-Receptor family-related gene*
HCV	*Hepatitis C Virus*
HIV	*Human Immunodeficiency Virus*
ICOS	*Inducible Costimulator* -
ICV	Imunodeficiência Comum Variável
IDP	Imunodeficiência Primária

Ig	*Immunoglobulin /* Imunoglobulina
ITP	*Immune thrombocytopenia*
IUIS	*International Union of Immunological Societies*
IVIG	*Intravenous immunoglobulin*
M	*Male*
NK	*Natural Killer*
NSAIDs	*Non-steroidal anti-inflammatory drugs*
OS	*Oral steroids*
PBMC	*Peripheral Blood Mononuclear Cell*
PCR	*Polymerase Chain Reaction*
PE	*Phycoerythrin*
PerCP	*Peridinin chlorophyll protein*
PID	*Primary immunodeficiency*
RA	*Rheumatoid arthritis*
SD	*Standard deviation*
SLE	*Systemic Lupus Erythematosus*
TACI	*Transmembrane activator and calcium-modulator and cyclophilin-ligand Interactor*
TRECs	*T-cell receptor-rearrangement excision circles*
T reg	*T regulatory /* T reguladora
WHO	*World Health Organization*

RESUMO EXTENSO

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A Imunodeficiência Comum Variável (ICV) é a imunodeficiência primária (IDP) sintomática mais frequente, tendo uma prevalência estimada em 1 / 25 000[1,2] entre a população ocidental. O diagnóstico definitivo de ICV baseia-se na diminuição de IgG, IgA e/ou IgM, pelo menos 2 desvio-padrões em relação ao normal para a idade, associada à deficiência de produção de isohemaglutininas e/ou de anticorpos específicos e após exclusão de outras causas primárias ou secundárias de hipogamaglobulinémia[3,4]. O espectro clínico da ICV é extremamente amplo no tipo de manifestações clínicas e sua gravidade. Para além das infecções recorrentes, mais frequentemente respiratórias e digestivas, as doenças autoimunes[5], a hiperplasia linfoide[6], em alguns casos com padrão granulomatoso, e a incidência aumentada de neoplasias hematológicas[7] são alguns dos problemas clínicos mais frequentes na ICV.

A prevalência de doenças autoimunes (DAIs) na ICV tem sido estimada entre 21%[8] a 50%[9,10,11], contrastando com os 5-7% calculados para a população geral[12] e sugerindo a existência de defeitos imunológicos favorecedores da autoimunidade na ICV.

Este intrigante aumento da incidência de DAIs mediadas por células e/ou por anticorpos, numa IDP predominantemente atribuída a défice de anticorpos constitui um paradoxo aparente e tem originado várias hipóteses visando a sua explicação[13].

A existência de uma predisposição genética para a autoimunidade em doentes com ICV é sugerida por estudos de *linkage* e tipagem HLA que têm demonstrado associações entre genes de susceptibilidade major para ICV e/ou défice de IgA e outros para DAIs[9,14].

Na ICV, como em outras IDPs, a infecção poderá constituir o elo entre a imunodeficiência e a autoimunidade[15]. A incapacidade de lidar com super-antigénios[16] e de eliminação de antigénios externos, secundária aos múltiplos defeitos da imunidade inata e adquirida descritos na ICV, pode levar à formação de anticorpos contra tecidos lesados pelos agentes

infecciosos ou por uma resposta inflamatória exacerbada aos mesmos, a reactividade cruzada entre tecidos do doente e antigénios estranhos ou à deposição de complexos imunes.

Entre os múltiplos defeitos identificados na diferenciação / função dos linfócitos B, salientam-se defeitos na maturação de células de memória $CD19^{+}CD27^{+}$ [10,17,18,19,20]. Warnatz *et al* e Ko *et al* demonstraram que doentes com maior deficiência de células B de memória *class-switched* têm maior prevalência de DAIs[18,20]. Contrariamente, Piqueras *et al* não confirmaram esta associação, mas verificaram maior prevalência de esplenomegália, proliferação linfoide e doença granulomatosa no grupo de doentes com maior deficiência na maturação de linfócitos B[19].

Múltiplos defeitos de imunidade celular estão também descritos na ICV, nomeadamente linfopénia T[21], sobretudo CD4 *naive*[22,23], para a qual podem contribuir a redução de progenitores mononucleares na medula óssea[24], deficiência de timopoiese[2,24,], deficiência de IL2[25] e IL7[26] e aumento da apoptose[27,28,29]. Outros defeitos funcionais na imunidade celular incluem ainda alterações na activação e proliferação T[8,30] e na produção de citocinas, estando descrito neste contexto um desvio $Th1^{2}$ e diminuição de citocinas Th2, nomeadamente IL4, IL5 e IL10[31, 32, 33, 34, 35].

A falência de mecanismos de indução e/ou manutenção de tolerância central ou periférica pode também contribuir para o aumento da incidência de DAIs na ICV.

As células T reguladoras, entre as quais as $CD4^{+}CD25^{high}$, estão envolvidas na manutenção de tolerância ao *self*, através da supressão activa da activação e expansão de células T auto-reactivas existentes à periferia de todos os indivíduos saudáveis[36]. As células T reguladoras estão também envolvidas não só na supressão de reacções alérgicas e reacção enxerto *vs* hospedeiro após transplante, mas também da resposta a infecções e tumores[37,38].

Com este estudo pretendemos verificar se uma deficiência quantitativa de $CD4^{+}CD25^{high}$ se poderia associar ao aumento da prevalência de DAI descrito na ICV, podendo este ser um

defeito universal ou definir um perfil particular de um subgrupo de doentes com expressão clínica / laboratorial de autoimunidade. Embora tivesse já sido anteriormente sugerida a existência de um compromisso funcional das células T supressoras na ICV e sua associação com o aumento de prevalência de DAIs[14], a hipótese de um defeito quantitativo de CD4^{+}CD25^{high} nunca antes tinha sido testada.

Sakagushi *et al* demonstraram que a depleção de células reguladoras CD4^{+}CD25^{+} resulta no desenvolvimento de DAIs em ratinhos[39]. O mesmo grupo demonstrou em animais que uma população *minor* de células T CD4^{+}CD25^{+} é crucial para o controlo de células T autoimunes *in vitro*[40,41]. Diversas AID foram induzidas em estirpes susceptíveis de ratinhos, em protocolos envolvendo remoção completa ou alteração do desenvolvimento das células T CD4^{+}CD25^{+}, nos quais a co-transferência de células T CD4^{+}CD25^{+} evitava o desenvolvimento de DAIs[38,41,42]. Adicionalmente, os ratinhos com deficiência primária de CD25 demonstraram ser susceptíveis a autoimunidade grave que podia ser evitada pela inoculação de células T CD4^{+}CD25^{+} de ratinhos singénicos[43]. No seu conjunto, estes dados sugerem que as células T CD4^{+} que expressam primariamente a cadeia α do receptor da IL2 (CD25) desempenham um papel importante na patogénese das DAIs.

Uma população com propriedades *in vitro* fenotípicas e funcionais idênticas foi posteriormente definida nos humanos, no sangue periférico, timo e sangue venoso umbilical de recém-nascidos saudáveis[44,45,46,47,48]. A capacidade supressora destas células foi preferencialmente associada às células T CD4 com maior intensidade de expressão de CD25 (CD4^{+}CD25^{high})[44] e num estadio final de diferenciação, sendo maioritariamente CD4^{+}CD25^{+}CD45RA^{-}CD45RO^{+} [44]. A população total CD4^{+}CD25^{+} contém uma proporção relativamente elevada de células T activadas, já que o CD25 é expresso transitoriamente à superfície de células T CD4^{+} não reguladoras após activação, não conferindo actividade supressora[44].

Muitos trabalhos têm procurado clarificar o papel das células T $CD4^+CD25^+$ na patogénese das DAIs no ser humano. Defeitos quantitativos e / ou funcionais têm sido descritos em diversas DAIs, embora os resultados sejam escassos e discrepantes. Diferentes autores encontraram uma diminuição do número de células T $CD4^+CD25^+$ circulantes na diabetes insulino dependente[49], hepatite autoimune[50] e lúpus eritematoso sistémico[51,52] e defeito funcional, mas não quantitativo, no síndrome poliglandular tipo II[53], esclerose múltipla[54] e diabetes autoimune[55,56]. Outros estudos não detectaram qualquer deficiência de $CD4^+CD25^{high}$ na *miastenia gravis*[57], esclerose múltipla[58], diabetes insulino-dependente[59] e síndrome de Sjögren[60]. Na artrite reumatoide foi encontrada maior quantidade de células T reguladoras, com actividade supressora mais intensa, no líquido sinovial de articulações inflamadas, em comparação com o sangue periférico dos mesmos doentes[61,62], um fenómeno com fundamento possivelmente equiparável ao aumento de células reguladoras $CD4^+CD25^+$ verificado na mucosa intestinal de doentes com doença inflamatória intestinal[61,62].

No presente trabalho, foi estudada uma população de 47 doentes com ICV seguidos no Hospital Vall d´Hebron, em Barcelona, com os objectivos de avaliar a frequência de DAI nesta população, caracterizar os doentes com ICV e DAI e comparar o seu perfil clínico e imunológico com o de doentes sem DAI. Pretendemos ainda avaliar a frequência de $CD4^+CD25^{high}$ em doentes com ICV e DAI em comparação com controlos saudáveis.

A caracterização clínica foi realizada através da revisão do processo clínico hospitalar e entrevista guiada com cada doente. Foram colhidos dados relativos à ICV, nomeadamente idade e tipo de apresentação, evolução clínica, idade de diagnóstico e de início de terapêutica substitutiva com imunoglobulina endovenosa (IGEV), para além da evolução de eventuais DAIs, incluindo tipo de DAI, idade de diagnóstico e respectivo tratamento. Foram ainda recolhidos dados laboratoriais, nomeadamente doseamento de IgG, IgA, IgM, subclasses de IgG e produção de anticorpos específicos na altura do diagnóstico de ICV. Em paralelo,

realizou-se uma avaliação laboratorial transversal desta população, incluindo IgG pré–infusional, hemograma e imunofenotipagem com quantificação por citometria de fluxo das populações B, NK, T CD4^{+}, T CD8^{+} e expressão de HLA-DR nas duas últimas subpopulações T. Em paralelo, foi feita a avaliação quantitativa da percentagem de CD25^{high} entre as células T CD4^{+}, por citometria de fluxo em amostras de sangue total com tripla marcação CD4 / CD25 / CD45-RO, cujos resultados foram comparados com os obtidos numa população controlo de 29 saudáveis.

Foram identificados 19 doentes (40,4%) com manifestações de DAI ao longo da sua evolução clínica. As DAIs detectadas foram Síndrome de Evans, trombocitopénia autoimune, anemia perniciosa, eritroblastopenia, artrite reumatoide, vitiligo, *alopecia areata*, psoríase, Síndrome de Sjögren, hepatite autoimune, doença de Crohn e hipotiroidismo primário. Cunninghan-Rundles *et al* descreveram DAI em 52 / 248 doentes[8], embora prevalências ainda mais altas tenham sido reportadas na literatura (28%[63] a 50%[10]). Tal como em outras séries, as citopénias autoimunes foram as DAIs mais frequentemente diagnosticadas (6 / 47)[10,64], seguidas da artrite reumatoide e anemia perniciosa (6,4%).

Verificámos um predomínio não significativo do sexo feminino, tanto no conjunto de toda a população com ICV, como entre os doentes com DAIs. A idade de início dos sintomas foi muito variável, mas em média ligeiramente mais precoce que em outras séries (15,6 ± 14,7 anos)[8,9].

As primeiras manifestações atribuíveis à imunodeficiência no grupo de doentes com DAI foram as infecções respiratórias recorrentes, seguidas da autoimunidade em oito doentes, nenhum deles sob terapêutica imunossupressora durante o ano que precedeu o diagnóstico de ICV. Apenas dois doentes estavam sob terapêutica imunossupressora (ciclosporina) aquando da realização da avaliação laboratorial transversal.

A idade média dos doentes com ICV e DAI era significativamente superior à dos doentes sem DAI, não só no momento da realização do estudo, como no aparecimento dos primeiros sintomas atribuíveis à ICV e no início da terapêutica com IGEV. Os doentes com DAI apresentavam demora média desde os primeiros sintomas até ao diagnóstico de ICV significativamente mais longa.

Não se verificaram diferenças na idade de apresentação ou de diagnóstico de ICV quando comparados doentes que tiveram manifestações de DAI como primeiros sintomas com os restantes, com outros tipos de apresentação. No entanto, os doentes com DAI, mas em que esta não foi a primeira manifestação de ICV tiveram demora média significativamente mais longa desde a apresentação até ao diagnóstico de ICV do que doentes sem DAI.

Verificámos que os doentes com DAI tinham mais frequentemente diarreia crónica não infecciosa e hiperplasia linfoide, sendo esta diferença significativa. As infecções respiratórias recorrentes e bronquiectasias, associadas frequentemente a tosse crónica e sinusite, e as gastrenterites infecciosas foram frequentes no conjunto de todos os doentes, no entanto sem diferença significativa entre os doentes com e sem DAI.

Atendendo aos níveis de referência de imunoglobulinas séricas para cada idade, a IgA e IgG no diagnóstico estavam diminuídas em todos os doentes com DAI, encontrando-se a IgM dentro dos valores de referência em cinco destes doentes. A IgG sérica no diagnóstico era significativamente superior em doentes com DAI, particularmente nos que tinham esta forma de apresentação inicial, quando comparados com doentes sem DAI.

Aquando da avaliação transversal todos os doentes estavam sob terapêutica com IGEV com doses e periodicidade muito variáveis (de 373 a 1360 mg/Kg/mês). A IgG pré-infusional, hemograma e as populações B, NK, T $CD4^+$, T $CD8^+$ não revelaram diferenças significativas entre os doentes com e sem DAI.

Os doentes com ICV e DAI apresentaram médias de frequências de células T CD4$^+$CD25$^+$ e de CD4$^+$CD25^{high} significativamente inferiores às dos doentes sem DAI e controlos saudáveis. O conjunto total de doentes com ICV apresentou também frequência média CD4$^+$CD25^{high} inferior quando comparada com a dos controlos, sendo esta diferença mais significativa no grupo dos doentes com DAI e mantendo significado estatístico após exclusão dos dois doentes sob terapêutica com ciclosporina.

A percentagem de células CD25^{high} entre as células T CD4$^+$ foi extremamente variável nos doentes e nos controlos, salientando-se o facto de a média das frequências de CD4$^+$CD25^{high} obtida no grupo dos controlos saudáveis (1,25 ± 0,26 %) ter sido muito semelhante à obtida por Baecher-Allan *et al* no trabalho utilizado como referência metodológica para definição da população CD25^{high} no presente estudo[44]. Naquele trabalho, as células CD4$^+$CD25^{high} foram estimadas em 1-2% da população T CD4$^+$, sendo definidas por citometria de fluxo, com dupla marcação CD4 e CD25, como uma *subpopulação que se destaca da população contendo CD4$^+$CD25^{low} e CD4$^+$CD25$^-$* [44].

Mais recentemente, diversos autores têm descrito outras formas de definir a mesma população[52,55,56,59,60], alguns deles com maior objectividade, sendo outros omissos em relação à metodologia, incluindo critérios de definição de elevada expressão de CD25[51,53,57], o que dificulta a comparação entre resultados. No nosso trabalho, tentámos minimizar a subjectividade do método escolhido através da quantificação de CD4$^+$CD25^{high} por um único investigador, sem acesso aos dados clínicos e aplicando os mesmos critérios em todos os doentes.

Na nossa população, as células CD4$^+$CD25^{high} eram maioritariamente de memória CD45RO$^+$, tanto nos doentes com ICV como nos controlos. Foi avaliada a expressão de HLA-DR nas células T CD4$^+$ e T CD8$^+$, não tendo sido encontradas diferenças nos valores absolutos ou percentagens de células T CD4$^+$, T CD8$^+$, CD4$^+$HLADR$^+$ ou CD8$^+$HLADR$^+$ entre doentes

com e sem DAI. Estes resultados e a ausência de correlação entre $CD4^{+}CD25^{high}CD45RO^{+}$ e essas subpopulações sugerem que as diferenças encontradas nas frequências de $CD4^{+}CD25^{high}$ não seriam apenas directamente dependentes de uma maior activação imunológica.

Outros marcadores têm sido associados às células T reguladoras $CD4^{+}CD25^{+}$ T, incluindo CD152 (Cytotoxic T Lymphocyte associated-Antigen 4 - CTLA-4), GITR (Glucocorticoid-Induced TNF-Receptor family-related gene), CD62L e o factor de transcrição FoxP3. Este último está descrito como essencial ao desenvolvimento e actividade supressora, tanto em ratinhos como em humanos[44,65,66] e controla a expressão de CD25 nas células T reguladoras, mas não nas células T activadas[67]. A adição de outros marcadores, nomeadamente FoxP3, para caracterização imunofenotípica enriqueceria muito a nossa avaliação.

O defeito da timopoiese em doentes com ICV, anteriormente mencionado, pode contribuir para as baixas percentagens de $CD4^{+}CD25^{high}$ nestes doentes, já que o timo é uma fonte primária desta subpopulação[68]. Por outro lado, a IL2 é importante tanto para indução de apoptose de células T auto-reactivas[15] como na expansão e manutenção da função imunossupressora das células CD4CD25 à periferia[38,69,70,71]. A deficiência de IL2 em sobrenadantes de culturas após estimulação com mitogénios já foi descrita na ICV[72,73] e tem sido atribuída à linfopenia[74], a qual paradoxalmente se associa com a expansão de $CD4^{+}CD25^{high}$ em diferentes contextos[75]. Na nossa população não encontrámos linfopenia ou diferenças significativas nas percentagens ou valores absolutos de linfócitos entre doentes com e sem DAI. Adicionalmente, não encontrámos correlação entre os valores absolutos de linfócitos e frequências de $CD4^{+}CD25^{high}$, tanto considerando o conjunto de todos os doentes, como avaliando os grupos de doentes com DAI e sem DAI separadamente. A avaliação da capacidade de produção de IL2 pelas células T seria interessante neste contexto.

Alguns autores têm sugerido a existência de variações quantitativas / funcionais das $CD4^{+}CD25^{high}$ em função da idade[60]. Gregg *et al* relataram um aumento progressivo da proporção de células $CD4^{+}CD25^{high}$ [76], interpretado como predominantemente derivado de expansão periférica, enquanto Tsaknaridis *et al* encontraram um declínio progressivo da actividade supressora das células $CD4^{+}CD25$ [77], sugerindo eventual relação com o declínio da função tímica. Na nossa população, como em outras séries[55,56], não foi encontrada correlação entre a idade e a frequência de $CD4^{+}CD25^{high}$, quando considerada toda a população de doentes com ICV e controlos. Esta análise foi metodologicamente muito relevante, em virtude de terem sido encontradas diferenças significativas entre as idades dos grupos de doentes com e sem DAI. Por outro lado, o facto de não existirem diferenças significativas nas distribuições por idade entre controlos e doentes, não sugere ser a idade o factor responsável pelas diferenças significativas na frequência de $CD4^{+}CD25^{high}$ entre estas duas populações. Curiosamente, foi encontrada uma correlação positiva significativa entre a idade e a percentagem de $CD4^{+}CD25^{high}$ no subgrupo de doentes com DAI. O estudo da actividade supressora seria extremamente interessante, no contexto da hipótese de Tsaknaridis[77].

Foi também encontrada diferença significativa na frequência de $CD4^{+}CD25^{high}$ quando comparados doentes com e sem diarreia crónica não infecciosa, sem diferença significativa na distribuição por idade entre estes 2 grupos. No contexto da doença inflamatória intestinal, outros autores descreveram diminuição das células $CD4^{+}CD5^{high}$ no sangue periférico[78], em simultâneo com aumento significativo das mesmas células na lâmina própria intestinal[79].

No nosso estudo, não foram encontradas diferenças significativas na frequência de $CD4^{+}CD5^{high}$ no sangue periférico, após estratificação dos doentes de acordo com presença / ausência de bronquiectasias, esplenomegália, proliferação linfoide ou granulomas.

Alguns estudos têm tentado estabelecer uma classificação da ICV que permita prever quais os doentes com potencial evolução para DAI, embora sem sucesso[10]. Propomos a deficiência quantitativa de células T $CD4^{+}CD25^{high}$ como um marcador útil à identificação de doentes com maior risco de desenvolver DAI. Atendendo à elevada sobreposição encontrada nas frequências de $CD4^{+}CD25^{high}$ entre doentes com e sem DAI e entre doentes e controlos, propomos que a quantificação seriada / prospectiva desta população seria provavelmente mais informativa do que determinações isoladas.

Em doentes seleccionados, o re-estabelecimento / indução de tolerância dominante poderia ser tentado *in vivo* através da estimulação da expansão de células T reguladoras e/ou fortalecimento da sua actividade supressora ou da sua indução *in vivo* ou *in vitro*[37,80,81]. Ensaios envolvendo números limitados de doentes e usando IL2 sintética[82,83,84] ou natural[85] demonstraram o seu potencial clínico, embora estudos envolvendo maior número de doentes, com seguimento mais prolongado e com objectivos clínicos bem definidos sejam necessários antes de ser considerada a sua aplicação na prática clínica.

Confirmámos, numa população de 47 doentes com ICV, a elevada prevalência de DAIs nesta entidade (40,4%), sendo a autoimunidade a forma de apresentação em 8/47 (17%) dos doentes estudados. Sugerimos o doseamento de imunoglobulinas aquando do diagnóstico de DAI, um procedimento acessível, económico e que pode influenciar opções terapêuticas decisivas nestes doentes em particular, nomeadamente o início de fármacos imunossupressores e esplenectomia.

Verificámos uma diminuição da frequência de células T $CD4^{+}CD25^{high}$ na população de doentes com ICV quando comparada com controlos, particularmente acentuada no subgrupo de doentes com DAI. Estudos prospectivos, envolvendo séries com maior número de doentes, e idealmente uma avaliação fenotípica e funcional mais detalhada das células T

$CD4^{+}CD25^{high}$ permitirão integrar de forma mais adequada os nossos resultados na patogénese da autoimunidade na ICV.

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ARTIGO CIENTÍFICO

CD4^{+}CD25^{HIGH} AND AUTOIMMUNITY IN COMMON VARIABLE IMMUNODEFICIENCY: SEARCHING NEW ANSWERS FOR AN OLD QUESTION

ABSTRACT

Background: Several mechanisms have been proposed to explain the high incidence of autoimmune diseases (AID) in Common Variable Immunodeficiency (CVID). We aimed to evaluate AID frequency within a CVID population and to characterize patients with AID. We also investigated whether a quantitative defect in the immunoregulatory population CD4^{+}CD25^{high} could be associated with increased prevalence of autoimmunity in CVID.

Methods: 47 patients with CVID on regular intravenous immunoglobulin substitution therapy were enrolled. Chart review and questionnaire-guided interview were used to collect clinical and laboratory data concerning presentation symptoms and clinical evolution, including AID. Serum immunoglobulins were quantified at diagnosis. A cross-sectional evaluation was performed before IVIG infusion, including serum IgG level, T, B and NK cell quantification by flow-cytometry in freshly whole blood. CD4^{+}CD25^{+} cells were simultaneously quantified in whole blood by flow-cytometry and compared with age-matched healthy volunteers.

Results: AIDs were diagnosed in 19 patients (40.4%) and autoimmune cytopenias were the most frequent. AID was diagnosed before CVID in eight patients, none on immunosuppressors in the year before CVID diagnosis. Patients with AID were older at the time of the present evaluation, at CVID diagnosis and at beginning of IVIG. They also exhibited higher prevalence of chronic non-infectious diarrhea and lymphoid hyperplasia and higher serum IgG at diagnosis. There were no significant differences in IgG pre-infusional levels and lymphocyte subpopulations between patients with and without AID. CD4^{+}CD25^{high} frequencies were significantly lower in patients with AID compared to those without AID and controls and in the whole group of CVID compared to controls.

Conclusions: Our results suggest that CD4^{+}CD25^{high} deficiency may possibly contribute to the high incidence of AID in CVID. More detailed and longitudinal evaluation of

$CD4^{+}CD25^{high}$ T cells in larger cohorts, including the use of additional markers and suppressor cells function assessment, will help to clarify this issue.

KEY-WORDS

Common variable immunodeficiency, autoimmune diseases, regulatory cells, $CD4^{+}CD25^{high}$

INTRODUCTION

Common variable immunodeficiency (CVID) was described for the first time in 1953 by Janeway [1]. It is nowadays the most frequently diagnosed primary immunodeficiency (PID) and the most common symptomatic congenital deficiency of the immune system. Its prevalence has been estimated as 1/25 000 in the western population [2,3] and worldwide it has been reported between 1/20 000 and 1/200 000 [4,5].

Diagnosis of CVID is established when there is marked decrease of two major serum immunoglobulin isotypes, usually IgG and IgM and/or IgA, over two standard deviations (SD) below mean values for age [6], in addition to impaired ability to specific antibody production after vaccination or exposure to a known infectious agent. Other primary or secondary causes for antibody deficiency should be excluded [7,8].

The most common clinical manifestations of CVID are recurrent pyogenic infections, usually by encapsulated bacteria and involving the sino-pulmonary tract, or otherwise unexplained chronic lung disease. Gastrointestinal manifestations are also frequent, either infectious, involving bacterial and protozoal agents or inflammatory conditions, leading to chronic diarrhea and malabsorption. In addition, CVID is associated with a remarkable incidence of autoimmunity and increased risk of gastric cancer and lymphoma [9] and other clinical presentations of benign lymphoid proliferation, including multiple adenopathies, splenomegaly and lymphocitic or granulomatous infiltration of lungs, lymph nodes or other sites [8,10,11]. In some cases, non-infectious complications dominate the clinical picture of CVID and have a significant impact on the overall severity of the disease.

Although included in the last update of the International Union of Immunological Societies (IUIS) classification of PIDs as predominantly an antibody deficiency [6], many other immunological defects have been reported in patients with CVID, involving both innate and

acquired immunity, including both humoral and cellular components, and mostly the interplay between all [3,12,13]. The possibility of extensive immunological heterogeneity underlying the wide diversity of presentation symptoms and clinical outcome has motivated recent works aiming to individualize groups of patients based on clinical features and match them to particular immunological defects. On the other hand, interest on a possible genetic basis of CVID has increased and, in the last 2 years, four monogenic defects associated with CVID have been identified: ICOS, TACI, BAFF-R and CD19 [2, 12].

Autoimmune disease (AID) prevalence has been estimated as 5-7% in the general population [14]. In one of the largest CVID series reported in the literature, Cunningham-Rundles *et al* refer to AID in 52/248 (21%) patients [15]. More recently, other authors have reported higher incidences ranging from 28% [4] to 50% [5,16,17]. Several mechanisms have been proposed to explain this high incidence of autoimmunity in CVID, although no immunological marker of autoimmunity has been identified in these patients.

Among different T cell sub-populations known to participate in the maintenance of tolerance, $CD4^+CD25^{high}$ lymphocytes have emerged as a major immunoregulatory population. Besides, evidence is now accumulating that regulatory T cells are also involved in the down-regulation of allergy, graft-versus-host disease and immune response to tumors and infections [18,19].

Although results are not consensual [20,21,22], quantitative [23,24,25] or functional [26,27,28,29] defects in $CD4^+CD25^{high}$ cells were found in some human AID. Even though a possible functional compromise of suppressor T cells promoting the growth of autoimmune clones in CVID has been suggested [30], the hypothesis of a quantitative defect in $CD4^+CD25^{high}$ had not been investigated in this context.

In the present work we aimed to evaluate AID frequency within a CVID population and to characterize a group of patients with CVID and AID, both from clinical and immunological point of view. We also investigated whether a quantitative defect in $CD4^+CD25^{high}$ in CVID

patients could be associated with an increased prevalence of autoimmunity. This could be either a defect of a particular homogenous group with clinical expression of AID or a common defect to the general population of CVID.

PATIENTS AND METHODS

A retrospective and descriptive study of AID prevalence was performed among a CVID population followed at Vall d´Hebron Hospital, a referral center for both pediatric and adult primary immunodeficiency patients in Barcelona, Spain. Clinical history from all patients was carefully reviewed concerning presentation symptoms and complications. A cross-sectional laboratory evaluation of these patients was also performed in the Immunology Department at the same institution. Institutional review board approval was obtained for this study.

Patients

The study included 47 patients (22 women / 25 men; mean age: 37.5 ± 15.9; range: 16-71 years old) attending Vall d´Hebron between January and March 2004 and with a mean follow up of 8.8 ± 6.1 years since CVID diagnosis. All patients were on regular intravenous immunoglobulin (IVIG) substitution therapy. Diagnostic criteria were according to WHO [6] and ESID recommendations [7]. Each patient presented a marked decrease in at least two out of the three major isotypes of serum immunoglobulins (IgG, IgA and IgM) by more than two SD below mean values for age on at least two separate occasions over one month. In most cases, including all patients with serum IgG level greater than 350 mg/dL, antibody deficiency was verified by means of decreased isohemaglutinins and / or antibody production to two or more vaccines, including tetanus, *Haemophilus influenzae* and pneumococcal vaccine. These patients were immunized with *Haemophilus influenzae* type B (Hib)-conjugated vaccine PedvaxHIB® and PNU-Immune23® polyvalent pneumococcal vaccine and titers of specific IgG before and four weeks after immunization were compared [31,32].

Subjects under the age of two years and all patients with known secondary causes of hypogammaglobulinemia at time of CVID diagnosis were excluded, namely hematological

disorders such as myeloma or non-Hodgkin lymphoma, HIV infection, nephrotic syndrome, exsudative gastroentheropathy, thymoma, chronic immunossupression or catabolic states due to malnutrition, treatment with drugs like hydantoin and gold salts. Patients with other known causes of primary hypogammaglobulinemia (hyper IgM syndrome, X-linked agammaglobulinemia, X-linked lymphoproliferative syndrome) or low peripheral B cell counts (<1% CD19^{+} cells) were also excluded.

Written informed consent was obtained from all patients before enrollment.

Clinical and laboratory data collection

Clinical and laboratory data concerning family history, first symptoms suggesting PID, CVID diagnosis, immunoglobulin replacement and clinical evolution, including complications / concomitant disorders and respective treatment have been collected by a single investigator by means of retrospective chart review and questionnaire-guided personal interview with all patients.

Vall d´Hebron's CVID follow-up protocol includes regular laboratory tests (hemogram, hepatic and renal function, PCR for HCV and HIV antigenemia, pre-infusion IgG level, stool and sputum cultures), imaging evaluation (annual abdominal ultrasonography and chest X-ray, biannual abdominal and thoracic CT) and annual lung function. Further examinations are performed when appropriate, in selected patients, in order to diagnose / treat concomitant diseases, mainly infectious, autoimmune or malignant. AIDs have been diagnosed by the assistant physicians or by the investigator, according to accepted criteria for each disease and based on typical clinical data and laboratory / imagiological exams and exclusion of other frequent diagnosis. The absence of autoimmune antibodies did not exclude AID diagnosis.

Serum IgG, IgA and IgM at diagnosis were quantified by nephelometry (reference values in individuals older than 16 years old were considered 850-1600mg/dL for IgG, 75-350mg/dL

for IgA and 58-250mg/dL for IgM). IgG subclasses were quantified by ELISA (reference values in adults were 261-1081mg/dL for IgG1, 112-408mg/dL for IgG2, 22-288mg/dL for IgG3 and 5-156 mg/dL for IgG4) [33]. Adequate responses to the 23-valent pneumococcal vaccine [32] and the Hib conjugated vaccine [31] were considered when, respectively, four-fold and two-fold increases in specific IgG titer were verified by ELISA.

Cross-sectional laboratory evaluation - CD4CD25^{high} quantification

A 10mL sample of peripheral blood in 0.05% EDTA was collected from CVID patients, prior to IVIG substitution. IgG level was quantified by nephelometry in this sample. Full blood counts, including white blood cell differential count were performed using a routine hematology analyzer in the Department of Pathology of Vall d´Hebron Hospital.

Peripheral blood mononuclear cells (PBMC) were isolated by Fycoll-Hypaque gradient. $CD4^+$ and $CD8^+$ T cells, B cells and NK cells were assessed in the Immunology Department by flow cytometry (FACSCalibur, Becton Dickinson Biosciences, San Jose, CA, USA) using the following monoclonal antibodies (Becton-Dickinson®): anti-CD3, anti-CD4, anti-CD8, anti-CD19 and anti-CD16 + anti-CD56. Anti-HLA-DR was used to assess CD4 and CD8 T cells activation.

$CD4^+CD25^+$ cells were quantified in whole blood in parallel experiment using 3 colour acquisition on a Fluorescense Activated Cell Sorter FACSCalibur (Becton-Dickinson®), with peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 (Becton Dickinson®), fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (Immunotech®), phycoerythrin (PE)-conjugated anti-CD45RO (Becton Dickinson®) and respective mouse isotype controls.

A single investigator with no access to clinical data acquired and analyzed all data using Cellquest software (Becton-Dickinson®).

Lymphocytes were gated according to forward and side scatter and a minimum of 10 000 events were acquired and analyzed. CD4^{+}CD25^{high} definition adopted in this work was based on Baecher-Allan *et al* [34], in which CD4^{+}CD25^{high} cells appear as *a tail to the right from the major population containing both CD4^{+}CD25^{low} and CD4^{+}CD25^{-} cells* (Figure 1).

CD4^{+}CD25^{high} percentage was defined as the percentage of CD25^{high} within gated CD4 positive T cells and its absolute number was calculated by multiplying this percentage by the number of CD4 positive T cells obtained in simultaneous sample. CD4^{+}CD25^{high} percentages obtained in patients were compared with those obtained in 29 age-matched healthy volunteers.

Statistical Analysis

Descriptive values of variables were expressed as the mean ± SD. CD4^{+}CD25^{high} percentages were compared using unpaired Student´s T test or Mann-Witney U test. Pearson´s correlation coefficient, Spearman´s rank correlation and Fisher exact test were used when appropriate to study the relationship between clinical and / or laboratory parameters. Statistical analyses were performed using Excel and Prism Graph Pad 4 Programs (GraphPad Prism, USA). Results were considered significant at a p value <0.05.

RESULTS

3.1 Autoimmune diseases in patients with CVID

Chart review and personal interview with the 47 patients revealed that 19 patients (40.4%) presented previous or present manifestations of AID (9 men and 10 women; mean age 46.5 ± 15.0 years old).

Twenty-six AID were diagnosed, including Evans´s Syndrome (1), immune thrombocytopenia (ITP) (5), autoimmune hemolytic anemia (1), pernicious anemia (4), eritroblastopenia (1), rheumatoid arthritis (3), vitiligo (1), *alopecia areata* (3), psoriasis (2), Sjogren's Syndrome (1), autoimmune hepatitis (1), Crohn's Disease (2) and primary hypothyroidism (1).

Table 1 details AID diagnosis, age at presentation and AID treatment of the 19 patients. Six patients had more than one AID, with autoimmune cytopenias being the most frequently diagnosed (26.3%). In eight patients (patients 12 to 19), AID was diagnosed before CVID diagnosis (mean delay 13.6 ± 10.3 years; maximum delay 29 years). After starting symptoms of AID, patients 13, 14, 15, 16, 17, 18 and 19 were treated with oral steroids during variable periods but not in the year before CVID diagnosis were made. Investigations that lead to CVID were mainly prompted either by frequent respiratory infections (patients 12, 13, 14, 15 and 19) or recurrent bouts of autoimmune cytopenias (patients 16 and 18). Patient 17 was diagnosed CVID when she was 14 years old. She presented with vitiligo and autoimmune hepatitis when she was nine years old. IgA deficiency was then diagnosed and small doses of oral steroids and azathioprine were prescribed during one year.

At the time of the present study laboratory evaluation (including $CD4^{+}CD25^{high}$), patients 6 and 13 were the only ones on immunosuppressive therapy - cyclosporine for Crohn's disease,

3.2 Other clinical features of patients with AID

First symptoms of CVID were recurrent respiratory infections in 11 cases and AID in the other eight, with mean **age at the beginning of symptoms** of 20.9 ± 15.6 and 21.2 ± 13.8 years old respectively, as described in Table 2. Mean **age at CVID diagnosis** was 46.5 ± 15.0 years old (minimum 10; maximum 58). There were no significant differences in age at the beginning of symptoms or at diagnosis between patients whose first symptoms were of AID and those who initially presented with upper or lower respiratory infections and developed AID during evolution (n=11).

Sixteen patients presented recurrent upper and lower respiratory infections and bronchiectasis during evolution in association with chronic productive cough in eight and sinusitis in 11 patients.

Twelve patients reported intermittent periods of diarrhea with no infectious cause identified in stool cultures and intestinal biopsies. In four of these patients lymphoid nodular hyperplasia was found in intestinal biopsy, possibly justifying chronic diarrhea. Eight patients had recurrent infectious diarrhea and *Giardia lamblia* was the most frequent cause. Only six patients did not present gastrointestinal symptoms.

Lymphoid hyperplasia, defined as the presence of splenomegaly and / or lymphadenopaties, was found in 12 patients. Patients 1 and 13 were splenectomized for uncontrolled autoimmune cytopenias at 39 and 13 years old, respectively, and presented lymphadenopaties in both cases. Three patients had granulomatous disease, which may have been underdiagnosed, as biopsies were not performed in all patients. There were no reports of malignancy during clinical evolution.

All patients were on IVIG replacement therapy, with highly variable doses and periodicity, individually adapted to each patient's weight and clinical condition (668 ± 402 mg/Kg/month; 373-1820 mg/Kg/month).

3.3 Immunological features of patients with AID

Levels of immunoglobulins and IgG subclasses at time of diagnosis of CVID in patients with AID are detailed in Table 3, which also shows hemogram, lymphocyte subsets evaluation and pre-infusional IgG cross-sectional results.

Three patients were diagnosed CVID before 18 years of age (patients 6, 15 and 17) and the remaining 16 after the third decade of life. Considering the normal range of serum immunoglobulins for each age, IgA and IgG were decreased in all patients, although IgG at diagnosis was above 350mg/dL in 12 of them. IgM was not decreased in five patients.

Hemogram within cross sectional study revealed anemia (hemoglobin <11g/dL) in patients 10 (pernicious anemia) and 13 (ferropenic anemia) and thrombocytopenia (platelets <100000/mm^3) in patient 3 with a previous diagnosis of ITP. Lymphopenia (lymphocytes <1000/mm^3) was detected in four patients and CD4 lymphopenia (<500/mm^3) in eight. An inversion of CD4/CD8 (<1) was found in five patients. All patients had more than 2% B lymphocytes, although four of them presented less than 100 cells/mm^3.

Serum IgG level obtained before immunoglobulin infusion was highly variable (mean 695.9 ± 181.5 mg/dL) and was under 500 mg/dL in two patients.

3.4 Comparison between patients with and without AIDs

Clinical and immunological features of patients with and without AID are compared in Table 4. Patients with AID were significantly older than patients without AID when this study was performed (p=0.0017, Figure 2) and at first symptoms of CVID, although this difference was not significant. Mean delay between first symptoms and CVID diagnosis was significantly longer in patients with AID (p=0.022) and patients with AID were significantly older both at CVID diagnosis (p=0.0022) as well as at the beginning of IVIG therapy (p=0.004).

When comparing patients with first symptoms of AID (n=8) with those with other types of clinical presentation (n=39) there were no significant differences in age at beginning of symptoms or at CVID diagnosis. Nevertheless, among patients that did not present initially with AID (n=39), mean delay until diagnosis was significantly longer in those that developed AID during clinical evolution (p =0.029).

Patients with AID presented significantly higher prevalence of chronic non-infectious diarrhea (p=0.015) and lymphoid hyperplasia (p=0.043) than patients without AID, but frequency of splenomegaly was not significantly different between these two groups.

Regarding laboratory evaluation, IgG serum level at diagnosis was remarkably lower in patients with no AID (p=0.0009). Grouping the 47 patients according to type of clinical presentation, IgG level at diagnosis was also significantly higher in the 8 patients who initially presented with AID than in those with other types of first symptoms (respectively 413 ± 87 mg/dL and 270 ± 146 mg/dL; p=0.002). There were no significant differences in recent pre-infusional IgG levels or hemogram counts between patients with and without AID.. Leukocyte differential count, lymphocyte subpopulations and HLA-DR expression both in $CD4^{+}$ and $CD8^{+}$ T cells were comparable in both groups, with no significant differences in absolute values or percentages.

3.5 $CD4^{+}CD25^{high}$

$CD4^{+}CD25^{+}$ T cells and $CD4^{+}CD25^{high}$ T cells were analyzed as illustrated in Figure 1 and results are shown in Table 5. $CD4^{+}CD25^{+}$ T cells frequency was significantly lower in patients with AID, both when compared with those without AID (p=0.0199) and with controls (p=0.041), as shown in Figure 3(A). When considering percentage of $CD4^{+}CD25^{high}$ within $CD4^{+}$ T cells, these differences were more significant, being these percentages lower in patients with AID both comparing with patients without AID (p=0.0016) and with controls

(p<0.0001). A significant difference was also found when evaluating the whole group of CVID and controls (p=0.011), with mean $CD4^+CD25^{high}$ lower in the first group, as shown in Figure 3(B). These differences in $CD4^+CD25^{high}$ frequency maintained statistical significance (p=0.0038, p<0.0001 and p=0.023, respectively) after exclusion of the two patients under treatment with cyclosporine.

A significant difference was found in current age between patients with and without AID (Figure 2). No correlation was found between age and $CD4^+CD25^{high}$ frequency in the control group or in the whole group of patients, but interestingly there was a significant positive correlation in the subset of patients with AID (Spearman r=0.47; p=0.04; Figure 4). No correlation was observed between the degree of lymphopenia and levels of $CD4^+CD25^{high}$, in spite of lymphopenia having been described to be associated, in different contexts, with $CD4^+CD25^{high}$ increase [35].

Simultaneous staining of CD4, CD25 and CD45RO showed that the large majority of $CD4^+CD25^{high}$ in the cohorts were in fact $CD45RO^+$ (more than 96%). T regulatory (T reg) cells have been described as mostly $CD45RO^+$ [34a], as it was seen in this population, both in CVID patients with and without AID and in controls. There was no correlation between $CD4^+CD25^{high}RO^+$ and lymphopenia, $CD4^+$ or $CD8^+$ absolute values or percentages within lymphocytes. $CD4^+CD25^{high}RO^+$ were also not correlated with absolute numbers or percentages of $HLA\text{-}DR^+$ cells within $CD4^+$ or $CD8^+$ T lymphocytes.

Correlation of $CD4^+CD25^{high}$ percentages with other clinical data was also studied. The only significant difference in levels of $CD4^+CD25^{high}$ was found when comparing patients with and without chronic non-infectious diarrhea ($CD4^+CD25^{high}$ respectively 0.697 ± 0.371% and 1.119 ± 0.711%; p=0.011), with no significant difference in ages between these two groups.

DISCUSSION

The high incidence of autoimmunity in primary immunodeficiencies represents an apparent paradox of immunology that has congregated growing interest among immunologists in the last years [36,37,38]. Several authors have suggested that autoimmunity and immunodeficiency are not mere contraries but different facets of a dysregulated immune system [39,40]. PIDs were once considered as limited to those clinical conditions with increased incidence or severity of infectious diseases, but nowadays it is clear that PIDs are also characterized by increased susceptibility to cancers (especially lymphomas), autoimmune diseases and in some cases, dysregulated inflammation due to abnormal infiltration of lymphocytes in tissues and organs [41]. AIDs are common manifestations not only in CVID but also in other PIDs, namely IgA and C2 deficiencies [41].

The high incidence of both cellular and autoantibody-mediated AIDs in CVID that is mainly characterized by a deficit of antibodies production suggests that more complex defects in immune system, beyond quantitative and qualitative defects in antibodies production [5], should underlie its high diversity in clinical manifestations. An increasing list of immune defects has been reported in the last years, including B and T cell defects besides B-T cooperation and innate immunity impairment. In most cases, these defects are not universal to the whole CVID population, but affect subgroups with varying degrees of severity. CVID is though presumed to congregate a heterogeneous group of disorders with separate etiologies and distinct clinical and immunologic features [6,42].

Several authors have tried to develop a classification of CVID patients based on immunological profile with clinical correlates. This would allow physicians to create follow-up protocols adjusted to each group of patients, optimizing the screening and diagnosis of complications and respective treatment. The identification of homogeneous groups of patients, from clinical and immunological point of view, would further allow pursuing genetic

investigation and, eventually, the identification of different entities amongst CVID. Patients with AIDs may constitute one of those homogenous groups and their extensive clinical and laboratory characterization may contribute to a better understanding of the etiopathogenesis of this situation.

We present a group of 47 patients with CVID followed at Vall d´Hebron Hospital. In our series, 19 patients (40.4%) presented with AID either before or after CVID diagnosis. Cunninghan Rundles *et al* reported AID in 52/248 (21%) patients from multiple institutions, excluding 7 with anti-IgA antibodies and no symptoms [15]. Nevertheless, higher incidences have been recently reported, ranging from 9/32 (28%) [4] to 20/40 patients (50%) [16]. This wide variability may be influenced by differences between CVID populations studied, but also by heterogeneity in AID diagnostic criteria used in each study. There are increased difficulties in the diagnosis of autoimmunity in CVID, since autoantibodies, that are usually decisive criteria in supporting AIDs diagnosis, may be absent as part of antibody secretion impairment [5,43]. Conversely, as in the general population, autoantibodies may be detected in absence of clinical AID in CVID patients [17]. Moreover, serological methods are of no value in patients under IVIG replacement therapy and therefore should not support AID in this context.

Autoimmune cytopenias were the most frequently diagnosed AIDs in our population, in agreement with reports from several authors[16,44]. In our series, autoimmune cytopenias were diagnosed in 6/47 patients (12.8%) of the whole population. This frequency is very similar to the prevalence of hematological autoimmune manifestations found in a series of 326 patients with CVID (11%), in which ITP has been the most frequent cytopenia [10]. A multi-center retrospective study in France involving 105 CVID patients described an even higher prevalence of ITP (20%)[43]. Incidences of both ITP and AIHA are strikingly lower in the general population, respectively 1.0-12.5 per 100 000/year [45] and 1-3 per 100 000/year [46].

Rheumatoid arthritis (RA) and pernicious anemia were the second most frequent diagnosis of AID in our population (6.4% each). Aseptic polyarticular arthritis that resembles RA, although frequently non-erosive, has been observed in 10-30% of CVID patients [30, 47]. Autoimmune arthritis is characterized by symmetric involvement of joints, most often the knees, ankles and hands; it is rarely destructive and rheumatoid factor and antinuclear antibodies are typically absent. Diagnosis of RA is difficult in CVID patients, as serological diagnosis of RA is not reliable and other causes of arthropathy should be excluded, namely infectious and amyloidosis. Presence of HLA DRB1*01 antigens was proposed as helpful in early RA diagnosis [48].

Many other AIDs have been described in patients with CVID, both involving AIDs that have been formerly associated with predominant humoral and cellular immunity. Some of the AIDs we found in our population had been previously reported in CVID patients, namely pernicious anemia [49], vitiligo [49,50], psoriasis[16] and inflammatory bowel disease[30]. In the literature, there are reports of many other AIDs in CVID patients, including juvenile rheumatoid arthritis [51], primary biliary cirrhosis [4,49], *alopecia totalis* [4,52], Systemic Lupus Erythematosus (SLE)-like syndromes [53,54], vasculitis [15], Insulin Dependent Diabetes Mellitus [5,52,55], celiac disease [56], Guillain-Barré Syndrome [47], myasthenia gravis [4] and autoimmune thyroiditis [52].

Considering the 47 patients we evaluated, there was a slight non-significant predominance of males (53.2%), which is not in agreement with any known preferential incidence of CVID in the male gender [5]. Other authors have reported that autoimmune phenomena in CVID patients are more frequent among female, which has been stressed by a study, in which 61% of the patients with AID were female[15]. In fact, when considering the whole population in that study, prevalence of autoimmunity was not significantly different between the 102 men (25.4%) and 146 women (27.4%). Similarly, in our series, AIDs prevalence among females (10/22 –

45.5%) was higher than among men (9/25 - 36%), although this difference was not significant.

In our population, age at beginning of symptoms attributed to CVID was quite variable; mean age at presentation was 15.6 ± 14.7 years old (minimum 4; maximum 51). First symptoms occurred earlier in our population when compared with other studies [5,15]. Cunningham-Rundles *et al* reported 248 patients with mean ages at symptoms beginning of 23 and 28, respectively in males and females [15]. Nevertheless, comparisons are complex concerning this parameter, as its evaluation is difficult based on retrospective studies that are frequently dependent on patients' awareness and memory and on the investigator's valorization of initial manifestations, which may be influenced by a previous diagnosis of CVID. Hermaszewski *et al* [29] and more recently Salzer *et al* [2] have reported a bimodal age at beginning symptoms, including two peaks, being the first one in the first decade of life and the second in early adulthood, although, less frequently, later presentations have also been described [16].

In our series, we found mean age at diagnosis of 28.4 ± 17.6 years and mean delay since first symptoms to diagnosis 12.8 ± 12.4 years, which is similar to what has been reported by other authors [4,5,15,43]. Patients with AID were not significantly older at the beginning of symptoms, but mean delay until CVID diagnosis was significantly longer in these cases (p=0.022). Interestingly, no significant differences were found in age at presentation or delay to diagnosis when comparing patients with AID as initial presentation (n=8) with those with other initial presentations (n=39), although a significant longer delay to CVID diagnosis was found in patients with AID during evolution but with a different initial presentation (n=11) than patients with no AID (p=0.029). In some cases, AID might have been missed as a possible PID manifestation thus contributing to a longer delay to CVID diagnosis.

AIDs are frequently the first manifestation of CVID or other primary antibody deficiencies, sometimes with no previous remarkable history of recurrent / severe infections [17,43] as it

happened in eight of our 19 patients with AID. Cunningham-Rundles *et al* reported that autoimmune hematological diseases appeared prior to CVID diagnosis in 54% of the cases described in a series of 326 patients [10]. In another study [43], ITP was first diagnosed in 62% of 21 patients with ITP and CVID and these two diseases were diagnosed simultaneously in four patients. Notably, a serum protein electrophoresis was performed in only one of the patients who were first diagnosed ITP and showed no abnormalities [43].

The use of immunosuppressive therapy in patients with first diagnosis of AID may disturb PID diagnosis. The etiopathogenesis of CVID in this context may be questioned as drugs used in AID treatment, namely sulfasalazine, gold salts, D-penicilamine, oral steroids and other immunossupressors may cause hypogammaglobulinemia [48]. More severe hypogammaglobulinemia [4] and longer period between immunosuppressive therapy and CVID diagnosis are usually mentioned to support the primary character of hypogammaglobulinemia. Both hypotheses must be considered in a critical evaluation of these patients [53,57,58,54,59,60,61]. Among our eight patients that initially presented with AID, none was on immunosuppressive therapy when CVID was diagnosed.

Immunoglobulin quantification when an AID is diagnosed has been a subject of controversy. The American Society of Hematology considered routine screening of serum immunoglobulins unnecessary and inappropriate in children but did not comment on adults in its practice guidelines for ITP [45]. Conversely, Heeney *et al* suggested quantitative measurement of serum immunoglobulins in children with autoimmune cytopenias, especially in those with a chronic or recurrent course [62]. Some authors have recommended immunoglobulin quantification when an AID is diagnosed, irrespective of patient's age, even in the absence of previous recurrent / severe infections suggesting PID, in particular when patients are going to start immunosuppressive therapy [17,43,55]. Given the increased incidence of AID in PID, we would support this proposal, as serum protein electrophoresis and

nephelometry are fairly inexpensive and accessible laboratory methods. Diagnosis of PID would have a major impact on these patients follow-up, particularly regarding therapeutic options that may include immunosuppressive drugs and splenectomy, and early / aggressive treatment of infections.

Possible benefits of an early IVIG substitution, with the aim of control and prevention of AIDs, namely autoimmune cytopenias, have been debated. It has been suggested that the decision to treat a patient with IVIG replacement should be based not only on the frequency and severity of infections, but also on the severity of autoimmune manifestations [63]. In particular, in X-linked agammaglobulinemia patients with AIDs, Etzioni A. reported that increased dose of IVIG could ameliorate their condition [39].

Based on the fact that ITP has been diagnosed in patients with CVID after starting IVIG treatment, Michel *et al* suggested that IVIG was notoriously ineffective in CVID-associated ITP when given at only 0.5 g/kg and did not influence its natural history when administered repeatedly, even at higher doses (1-2 g/kg, every 3 weeks) [43]. In opposition, Cunningham-Rundles *et al* reported that 30/35 (86%) patients with CVID developed hematologic AID either before or concurrent with CVID diagnosis and institution of IVIG ($p<0.0001$) and thus suggested that IVIG replacement therapy diminishes the occurrence of those conditions [10]. Moreover, Bloch Michel *et al* suggest benefits of substitutive treatment with IgG in the control of thrombocytopenia after corticotherapy [5].

In the literature, there are no controlled randomized studies showing the benefits of an early IVIG start relating to autoimmunity control and IVIG immunomodulatory activity has been claimed to immunomodulatory doses that are far above replacement doses currently used in CVID patients. Therefore, many groups tend to delay IVIG start while patients are free of severe or recurrent infections [62].

In our series, cytopenias and other AID evolution were benign most cases. The majority did not require systemic immunosuppressive therapy, except for occasional oral steroids, similarly to what has been reported by Warnatz *et al* [16]. Only patients 6 and 13, both with refractory Crohn's disease, were on current immunosuppressive therapy (cyclosporine) when evaluated. In other series, patients with cytopenia and unsuspected CVID had a more severe clinical course, characterized by chronic and recurrent cytopenia[16].

Besides the older age at diagnosis, we found that patients with AID presented significantly higher prevalence of chronic non-infectious diarrhea and lymphoid proliferation than patients without AID, although splenomegaly incidence was not significantly different between these two groups. Regarding laboratory evaluation, no significant differences were found between the two groups, except for patients with AID presenting higher mean IgG at diagnosis than patients without AID. IgG at diagnosis was even higher in patients initially presenting with AID. The high values of serum IgG frequently found in autoimmunity may possibly have contributed to the delay in CVID diagnosis in these patients.

Different hypotheses have been raised to explain the increased incidence of AIDs in CVID[41]:

1 – Genetic predisposition to autoimmunity. Although most cases of CVID are sporadic, about 10-25% of the patients report family history of humoral PID, including CVID, displaying either autossomal dominant or recessive modes of inheritance [2,4,64]. There is obvious familiar clustering of IgA deficiency and CVID, suggesting that genetic factors play an important part in CVID genesis[3]. Genetic linkage and haplotype analysis have shown that IgA deficiency and CVID share a major susceptibility locus in the HLA-DQ-DR haplotype on chromosome 6 [3,64,65], a region where alleles have been associated with SLE and celiac disease [30]. CVID has also been significantly linked to the haplotype HLA-A1-B8-DR3, which is associated with autoimmune disorders such as SLE [5].

2 - Infection as the link between immunodeficiency and autoimmunity [40]. Defective processing and clearing of external antigens from mucosal surfaces and abnormal handling of superantigens[66] may result in chronic inflammation and eventually in end-organ deposition of immune complexes, formation of anti-tissue antibodies or in cross reactivity between normal tissues and foreign antigens. Chronic EBV infection and increased exposure to organisms sharing epitopes with host constitution (molecular mimicry) may contribute to the activation of auto-reactive T cell clones [30].

Many immunological defects have been reported in CVID patients that may help understanding their inability to clear external antigens including a possible genetic predisposition to abnormal antigen handling [67].

A disturbed B cell function, with both early [68] and late B cell differentiation defects [69] including defects in up-regulation of CD70 and CD86 in naïve B cells [70,71], in signaling [72], somatic hypermutation [73,74,75] and impaired antibody affinity maturation [75] has been demonstrated in different studies. Different authors have reported B maturation defects with reduced populations of $CD27^+$ memory cells and lack of $IgD^-IgM^-CD27^+$ class-switched memory B cells and plasma cells[76] and increased percentages of undifferentiated B cells in peripheral blood of patients with CVID [77, 78, 79,80].

Besides humoral deficiency, T cell function compromise affects a large proportion of CVID patients [13,15,81]. T cell help is required for successful B cell maturation and impaired expression of T cell surface molecules may be responsible for the failure of B-cell differentiation and for the inability to generate a proper immune response, thus converting CVID more properly in a combined PID. In some patients, B cells secrete normal amounts of immunoglobulins when appropriately stimulated *in vitro*, suggesting that T cell dysfunction leading to inadequate B cell help, plays an important pathogenic role [82]. Vlková *et al* have

recently focused on mutual relations in T and B lymphocyte abnormalities in CVID and proposed that these are partially related to each other [83].

Decrease in absolute numbers of T cells has been described in approximately one third of CVID patients[84], due to reduced CD4$^+$ T cell subset, mostly naïve CD4$^+$CD45RA$^+$ [85,86] but also antigen-specific memory CD4$^+$ [87,88]. Inversion of CD4$^+$/CD8$^+$ ratio is frequent in CVID [84,89]. Many explanations have been proposed to T lymphopenia in CVID including deficient thymopoiesis [3] and IL2 production [90], abnormalities in IL7-mediated lymphocyte homeostasis [91] and/or increased apoptosis due to persistent antigen activation following infections [92,93], increased oxidative stress in CD4$^+$ cells [94] or spontaneous apoptosis, associated with increased CD95 expression in CD4$^+$ and CD4$^+$CD45RA$^+$ subsets [93].

De Vera *et al* reported a significantly increased level of T-cell receptor-rearrangement excision circles (TRECs) in a group of patients with CVID compared with age-matched controls, although with an accelerated decline of TREC levels with age, both in CD4$^+$ and CD8$^+$ T subsets [95]. This may be in association both with a more rapid reduction of thymic output in CVID individuals with age and/or with enhanced cellular activation and proliferation in CD4 and CD8 peripheral compartment. Isgro *et al* reported a reduction of CD31$^+$ recent thymic emigrants in a group of patients, in which decreased numbers of CD4$^+$ T cells were present in a large proportion of patients[96]. Differences between these studies have been partially attributed to the heterogeneity of populations and different methods employed.

Isgro *et al* have also shown a reduced content of primitive progenitors in bone marrow mononuclear cells of CVID patients, besides abnormal stromal cell composition and cytokine production with increased TNFα production and decreased IL2 production [96].

T cells functional defects include decreased T cell activation and proliferation [15,97] dependent on impairment of early signaling events [98,99,100,101] or integration of activating signals derived from TCR and co-stimulatory molecules [102], both in CD4$^+$ and CD8$^+$ T cells. In a subgroup of

patients with impaired T cell proliferation, predominance of $CCR7^-$ effector-memory T cells was reported [103]. $CCR7^-$ T cells are a subset of tissue-homing, memory T cell population with reduced proliferative capacity, IL2 secretion and CD40L expression. Reduced expression of cell surface molecules in some CVID patients, namely CD40L [104], attractin [105] or L-selectin [106] has also been reported.

Defects in innate immunity may additionally contribute to reduce external antigens clearance. Defective differentiation and maturation of dendritic cells, with decreased expression of co-stimulatory molecules CD80, CD86 and HLA-DR and impaired IL12 production [107,108,109] were described in some patients with CVID. Absolute and relative decreases in NK cell numbers [110] and impaired NK-mediated cytotoxicity [3] have also been reported. Phagocytosis by monocytes [111] may be compromised by defective opsonization mediated by Fc, complement receptors CR1 and CR3. Low producing coding alleles and promoter haplotypes for mannose binding lectin were correlated to an early age of disease onset and increased autoimmune disease incidence[67].

3 - Increased incidence of AID as part of immune dysregulation in CVID. Cytokine dysregulation has been reported with Th1 skewing [3], enhanced IFNγ production [3] and decreasing of production of Th2 cytokines, namely IL4, IL5 and IL10 [112,113,114,115,116]. Conversely, other authors have reported increased IFNγ production both in $CD4^+$ and $CD8^+$ lymphocytes [117].

IL2 has been reported as important in triggering the apoptosis of auto-reactive T cells [40]. A general reduction in IL2 secretion into culture supernatants following mitogenic stimulation of cultured CVID T cells is well known [112,118]. This decrease may reflect the reduction in $CD4^+$ T cells and particularly in $CD45RA^+$ cells in CVID, as intracellular production of IL2 by T cell following mitogenic stimulation is normal [117].

In addition, persistent activation of TNF system, described in a subgroup of patients [119], may contribute to autoimmune disorders and granuloma formation.

Cell subpopulations equilibrium disturbances also contribute to immune dysregulation in CVID. Recent work has shown that patients with a more profound lack of isotype switched memory B cells are more likely to develop autoimmunity [16,79,120]. Warnatz *et al* reported that patients with reduced numbers of switched memory B cells CD19^{+}CD27^{+}IgM^{-}IgD^{-} (<0.4% of total lymphocytes) had increased frequency of splenomegaly and autoimmunity[79] and could be further subdivided in a group with more than 20% CD19^{high}CD21$^{lo/neg}$, comprising preferentially patients with splenomegaly and autoimmune cytopenias and a group with less pronounced expansion of these cells [16]. Ko *et al* found higher rates of autoimmune and granulomatous disease in patients with increased proportion of immature B cells, which would possibly contribute to their enhanced autoantibody production [120,121]. In contrast, Piqueras *et al* found no differences in prevalence of AID between CVID patients classified by switched / non-switched memory B cells frequency, but lack of these memory cells was associated with higher prevalence of splenomegaly, lymphoid proliferation and granulomatous disease [80]. Bloch-Michel C *et al* [5] have divided CVID patients in 2 groups according to T lymphocyte activation degree, being group I with no AID or organomegalies and inactive T lymphocytes and group 2 with AID and/or organomegalies besides activation of T lymphocytes. The concurrent heightened susceptibility to AIDs and lymphoid proliferation in some series, including ours, has prompted the hypothesis that a common environmental antigen could trigger these manifestations [43] and remains an interesting topic for further investigation.

4 - Breakdown in central and / or peripheral mechanisms of tolerance induction or maintenance. Failure in central tolerance mechanisms may lead to the persistence of autoantibodies by different mechanisms, including abnormal somatic hypermutation, failure

to delete self-reactive clones [73,122] or defective differentiation and maturation of dendritic cells which may compromise competent induction of immune tolerance through interactions with T and B cells[3]. Auto-reactive T cells are known to be present in the periphery in healthy individuals, as they escape thymic clonal deletion and induction of anergy. Regulatory T cells are involved in the maintenance of peripheral self-tolerance by actively suppressing the activation and expansion of auto-reactive T cells [123]. The earliest experiences to suggest the existence of thymic generated specific regulatory T cells were by Nishizuka and Sakakura [124]. Sakagushi *et al* denominated these cells as the $CD4^+CD25^+$ natural T reg and have shown that depletion of $CD4^+CD25^+$ suppressor cells results in the onset of systemic AID in mice [125]. The same group later showed in animals that a minor population of $CD4^+CD25^+$ T cells was crucial for the control of autoimmune T cells *in vivo* [126,127]. Many experimental organ-specific AIDs were induced in susceptible strains of mice by protocols that resulted in the complete removal or delay of the development of $CD4^+CD25^+$ T cells and the co-transfer of $CD4^+CD25^+$ T cells prevented the development of AID [19,127,128]. Also CD25 deficient mice were demonstrated to be prone to severe autoimmunity that could be prevented by the inoculation of $CD4^+CD25^+$ T cells from syngenic mice [129]. These accumulated data have strongly suggested that $CD4^+$ T cells that naturally co-express the α-chain of the IL2 receptor (CD25) play an important role in the pathogenesis of AIDs.

A population with identical phenotypic and functional properties *in vitro* was later defined in humans in peripheral blood, thymus and in umbilical venous blood from healthy newborn infants [34,130,131,132,133], preferentially residing within those $CD4^+CD25^+$ T cells with brightest expression of CD25 [34]. Despite the growing interest in $CD4^+CD25^+$ T cells role in the emergence of AIDs in animal models, very limited and controversial information is available on their role in the pathogenesis of human AIDs. Quantitative or functional defects in

$CD4^{+}CD25^{+}$ have been pointed as possibly involved in different AIDs, although some discrepancies were found between published reports.

Different authors found a decrease in the number of circulating $CD4^{+}CD25^{+}$ in autoimmune diabetes [23], autoimmune hepatitis [24] and SLE [25,134] and functional impairment with normal numbers of circulating $CD4^{+}CD25^{+}$ T cells was detected in polyglandular syndrome type II [26], multiple sclerosis [27] and autoimmune diabetes [28,29]. Other studies have failed to detect any deficiency in $CD4^{+}CD25^{high}$ in myasthenia gravis [20], multiple sclerosis [21], autoimmune diabetes [22] and Sjögren's syndrome [135]. Interestingly, in rheumatoid arthritis, higher numbers of regulatory T cells with increased suppressive activity were found in synovial fluid from inflamed joints compared to peripheral blood [136,137], similarly to the reported increase of $CD4^{+}CD25^{+}$ T reg in intestinal mucosa of patients with inflammatory bowel disease [138]. Sun *et al* suggested that in patients with myasthenia gravis, decrease in circulating regulatory T cell frequency may be associated with disease activity [139], as found by Crispin *et al* in SLE [25], but this is not consensual [134].

Human $CD4^{+}CD25^{high}$ T cells are anergic to *in vitro* stimulation and strongly suppress the proliferation of responder T cells upon culture [140]. *In vivo*, the mechanisms involved in T reg mediated suppression remain to be determined. It is accepted that suppression by $CD4^{+}CD25^{+}$ T reg may be exerted by different means, depending on the microenvironment and on the pathologic context [18,141,142]. Different AIDs may utilize different pathways to disease hence in some the dysfunction of $CD4^{+}CD25^{high}$ regulatory cells may play a more prominent role [22] that would possibly explain the heterogeneous results obtained in human diseases. The majority of research on $CD4^{+}CD25^{+}$ T cells has focused on their effects on T cell populations but they have also been shown to have some effects in B cell function. $CD4^{+}CD25^{+}$ T cells inhibited B cell proliferation induced by lipopolysaccharide *in vitro* [134] and prevented the activation of anti-DNA antibodies producing B cells in a transgenic system [143]. Moreover,

activated $CD4^+$ T helper cells presumably provide stimulatory signals to relevant self-reactive B cells, rescue them from apoptosis and stimulate them to form autoantibodies [144] and $CD4^+CD25^+$ may down-regulate this T cell mediated production of self-reactive antibodies in an adoptive transfer system [145].

We hypothesized that $CD4^+CD25^{high}$ deficiency could contribute to the documented high incidence of autoimmunity in CVID, either as a defect of a particular/ homogeneous group of patients or as a more universal CVID defect. Any genetic abnormality or environmental insult could favor the emergence of autoimmunity if it would tip the balance between T reg cells and self-reactive T cells toward the dominance of the later.

In our population we found significantly lower $CD4^+CD25^{high}$ frequencies in the group of CVID when compared to controls and in patients with AID when compared with those without AID. These results favor the hypothesis that impairment of $CD4^+CD25^{high}$ is a common defect in CVID patients. The group of patients with AID had significantly lower $CD4^+CD25^{high}$ frequencies suggesting this as one of the possible defects underlying their high susceptibility to autoimmunity.

Other groups have investigated on $CD4^+CD25^{high}$ both in animal and human studies and progresses in the area have revealed new aspects on their phenotype and function. Nevertheless, methodological differences across different studies, namely different staining and/or gating strategies and different depletion methods [22,146], may contribute to the heterogeneity in results concerning $CD4^+CD25^+$ frequency / function and turns their comparison into a difficult issue.

Different methods for defining *high* intensity of CD25 expression may also influence $CD4^+CD25^+$ T reg quantification. Baecher-Allan *et al* initially defined $CD4^+CD25^{high}$ as a *tail to the right from the major population containing both* $CD4^+CD25^{low}$ *and* $CD4^+CD25^-$, as illustrated in their paper [34]. We decided to adopt this definition, as Baecher-Allan *et al*

previously showed data supporting the suppressive ability towards co-cultured CD4^{+}CD25^{-} T cells of CD4^{+}CD25^{high} population obtained with their method [34]. In order to minimize subjectivity in the present work, one single investigator performed all CD4^{+}CD25^{+} cytometry evaluations, including acquisition and analysis, with no access to clinical data and applying the same uniform criteria to all patients.

In our study, CD25^{high} percentages within CD4^{+} T cells were highly variable both in patients and controls and there was a visible overlap between patients with and without AID and controls, although statistical evaluation found significant differences when comparing cohorts' results. Baecher-Allan *et al* estimated CD4^{+}CD25^{high} in 1-2% of the total CD4^{+} population [34] and in our study, based on his method, CD4^{+}CD25^{high} mean percentage in healthy donors was 1.25 ± 0.36%, quite similar to data from other studies applying this methodology [27].

Lately, other authors have defined CD4^{+}CD25^{high} T cells as those CD4^{+} T cells whose CD25 positivity exceeded the level of CD25 positivity seen in the CD4^{-} T cells [22], as those having intensity of fluorescence of CD25 expression exceeding 100 [28,29,134,135], as the top 2% of the CD25 staining CD4^{+} T cells [28] or even do not state the method that has been used for defining high expression of CD25 [20,25,26]. Moreover, CD25^{high} frequencies are variably expressed within CD4^{+} or lymphocyte gate in different studies [22] and, again, some authors do not state which percentage is used [32]. Finally, some authors report on CD4^{+}CD25^{+} T reg frequency without expressing if they are referring to CD25^{high} expression or to all CD25 positive T cells [18]. Whole CD4^{+}CD25^{+} contain a relatively high proportion of previously activated T cells, rather than naturally occurring CD4^{+}CD25^{high} T reg cells, as CD25 is transiently up regulated on non-regulatory CD4^{+} T cells upon activation and does not confer suppressive activity by itself. Therefore not all CD4^{+}CD25^{+} cells are considered to be regulatory T cells.

$CD4^+CD25^{high}$ population includes the majority of cells with demonstrated suppressive capacity [34].

$CD4^+CD25^+$ T reg are believed to be in late stage of differentiation and are mainly found within the $CD4^+CD45RA^-/RO^+$ T cells, thus displaying a memory T cell phenotype [34]. Concordantly, in our study $CD4^+CD25^{high}$ T cells were mostly $CD45RO^+$, both in CVID patients with and without AID and in controls. The only activation marker that was evaluated in our population was HLA-DR expression. We found no significant differences in absolute number / percentages of $HLA\text{-}DR^+$ cells within $CD4^+$ or $CD8^+$ T lymphocytes between patients with and without AID and there was no correlation between $CD4^+CD25^{high}CD45RO^+$ frequencies and those subsets, further supporting that $CD4^+CD25^{high}$ differences between groups were not directly dependent on immunologic activation.

Other markers have been linked to $CD4^+CD25^+$ T reg, including CD152 (Cytotoxic T Lymphocyte associated-Antigen 4 - CTLA-4) [147], Glucocorticoid-Induced TNF-Receptor family-related gene (GITR) [148,149], CD62 L[150] and the Forkhead transcription factor (FoxP3) [151, 152,153,154].

FoxP3 was recently reported to be essential for the development and suppressive activity both in mice and human $CD25^+$ T reg cells [34,155,156] and it controls CD25 expression in natural T reg cells but not in activated T cells in general [151]. FoxP3 quantitative expression was correlated with functional suppression in the peripheral $CD4^+CD25^+$ T cell compartment in multiple sclerosis patients [157]. FoxP3 mutations underlie a fatal autoimmune lymphoproliferative disorder in humans, termed immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) Syndrome [158,159]. Considering that the development of natural T reg cells is at least in part genetically programmed, it has been suggested that autoimmunity might be considered in part as a primary T cell deficiency [19], eventually another way to close the circle between immunodeficiency and autoimmunity.

The use of additional markers that allow a more detailed quantification, like FoxP3, might improve the evaluation of the role of CD4^{+}CD25^{+} T reg in CVID and AID. After we presented our preliminary results [160], Horn *et al* have presented data on FoxP3^{+}CD25^{high} regulatory T cells quantification in 48 patients with CVID [161]. In this study, no significant differences were found in patients as compared to healthy controls. Although patients were not divided according to AID diagnosis, the authors reported that only one patient out of 17 with AID, presented T reg frequency below the 5th percentile. Both differences in populations' demographic or clinical characteristics and in lab methods used may have contributed to this disparity in results between these two studies.

Even though no other markers were used in our study, some authors have considered high expression of CD25 as a good marker for natural CD4 T reg [19,154], when cautiously analyzed. In addition, the differences we found in CD4^{+}CD25^{high} frequencies between patients with AIDs and both controls and patients with no AID were clearly significant and, notably, these differences in CD4^{+}CD25^{high} maintained statistical significance after exclusion of the two patients under treatment with cyclosporine. This analysis was performed because there is limited data available on how calcineurin inhibitors influence regulatory cells development and function *in vivo* [162].

Defective thymopoiesis in CVID, previously mentioned [95,96], may contribute to the low percentages of CD4^{+}CD25^{high} T reg, as thymus is a primary source of a subpopulation of these CD4^{+}CD25^{+} T reg cells [163]. Nevertheless, mechanisms regulating this production remain unclear [18] and in a study involving myasthenia gravis patients, thymectomy showed no obvious influence on the frequency of CD4^{+}CD25^{high} [20].

Molecular defects underlying immunodeficiency may interfere with the establishment or maintenance of self-tolerance by interference with expression of cytokines such as IL2, whose

deficiency has been reported in CVID and which is important in both triggering the apoptosis of auto-reactive T cells [40] and in the maintenance of $CD4^+CD25^+$ T reg in the periphery [19]. Although $CD4^+CD25^+$ T reg typically do not produce IL2, they depend on this cytokine signaling for development in the thymus, expansion in the periphery and activation of their immunosuppressive function [164,165,166]. Inhibition of IL2 production, for example by cyclosporine, may reduce natural T reg cells in the periphery by affecting their survival [19,21] and *in vivo* neutralization of IL2 reduces the peripheral and thymic frequency of T reg but not other T cells and causes AID in mice [167].

IL2 deficiency in CVID has been interpreted as a result of lymphopenia [112,117], which curiously has been described in association with $CD4^+CD25^{high}$ expansion in different contexts [35]. Regulatory cells are thought to play a protective role in situations where lymphopenia driven proliferation generates autoimmune clones selectively controlling their expansion and type of response [168].

In our population we did not find lymphopenia or significant differences between lymphocyte percentages or absolute counts between patients with and without AID. Moreover, we found no correlation between lymphocyte counts and $CD4^+CD25^{high}$, both when considering all patients or dividing them according to clinical history of AIDs. Evaluation of T cell ability to produce IL2 would be interesting in this context.

Correlations between low $CD4^+CD25^+$ T regulatory cells and impaired IL12 production [25] and data reporting that CD80 and CD86 expression in dendritic cells influence on $CD4^+CD25^+$ T reg mediated suppression [169] may also suggest a possible interference of other previously mentioned CVID immunological defects in $CD4^+CD25^+$ frequency and / or function [25,169].

Age-dependent variances in $CD4^+CD25^{high}$ frequency or function have been reported [135]. Gregg *et al* reported a progressive increase in $CD4CD25^{high}$ cells proportion with age, which was interpreted as predominantly derived from peripheral expansion, without significant

difference in suppressive efficiency [170]. On the other hand, Tsaknaridis *et al* found a decline in suppressive activity of CD4^{+}CD25^{+} T cells with age and hypothesized that this could be influenced by age dependent decline in thymic function [171]. Taking into account that significant differences were found between patients with and without AID both in age and CD4^{+}CD25^{high} percentages, it would be relevant to exclude the age contribution to this result. In our population, as occurred with others [28,29], we found no correlation between age and CD4^{+}CD25^{high} frequency when evaluating the whole group of patients and controls. Control and CVID cohorts have been age - matched, which further argues against age as a relevant factor to explain lower CD4^{+}CD25^{high} frequencies in patients. Interestingly, we found that within the subset of patients with AID, CD4^{+}CD25^{high} frequency increased with age. Tsaknaridis hypothesis may not be excluded by our study, as suppressive activity was not evaluated.

Relationship between CD4^{+}CD25^{high} frequencies and other clinical characteristics besides autoimmunity were also investigated and a significant difference was found when comparing CD25^{high} proportion within CD4^{+} T cells between patients with and without chronic non-infectious diarrhea. In other studies, CD4^{+}CD25^{high} T cells were decreased in the peripheral blood of patients with active inflammatory bowel disease [172] but significantly increased in intestinal *lamina propria* although retaining similar regulatory activity when compared with those from normal individuals [138].

WE found no significant differences in CD4^{+}CD25^{high} T cells frequencies when dividing patients according to the presence of bronchiectasis, splenomegaly, lymphoid proliferation or granulomas.

Some studies have been designed in order to establish a classification of CVID patients that allows predicting those who will develop AIDs, which so far has not been achieved [16]. We

propose $CD4^+CD25^{high}$ deficiency as a marker to identify those patients with increased risk for autoimmunity, although deficiency or dysfunction of natural T reg cells *per se* cannot determine which organs or tissues are to be targeted by the triggered autoimmune responses [126]. Considering the important overlap that we observed in $CD4^+CD25^{high}$ frequencies both between patients with and without AID and between patients and controls, we should probably look at the prognostic value of longitudinal individual values, instead of determining strict cut-offs.

Additional therapeutic strategies may be used in selected patients in association with IVIG replacement [173]. In patients with AID and $CD4^+CD25^{high}$ deficiency, the reestablishment or newly establishment of dominant tolerance could be tried, either by helping naturally present T reg cells to expand, strengthening their suppressive activity or by induction of adaptative T reg [18,174,175]. $CD4^+CD25^{high}$ cells were effectively expanded *in vitro* in presence of high concentrations of exogenous IL2 while retaining their suppressive activity [175].

Initial trials with only few patients using synthetic [177,178,179] or natural human IL2 [180] showed some potential clinical benefit, although further work is needed, with longer and larger studies and more clinical endpoints, particularly concerning AIDs. *In vitro* and *in vivo* treatment with steroids was found to up regulate FoxP3 expression in $CD4^+$ lymphocytes of healthy donors and in asthmatic patients [176] and has been noted to promote T reg cell development and function.

These alternative therapeutic strategies require further data before considering translation of these studies to the clinical practice.

In summary, in our population, patients with CVID presented mean $CD4^+CD25^{high}$ frequencies lower than healthy controls and this difference was more pronounced in the subpopulation of patients with AID. This may be one more piece to join the many pieces that

have been collected from the complex puzzle of CVID. It is not clear which of the T and B cell abnormalities are possibly causative, which are secondary and which are only epiphenomena. Moreover, some of the abnormalities have been detected in subpopulations of patients and do not constitute a universal defect in CVID patients.

Our results should prompt us to more detailed phenotype and functional evaluation of CD4^{+}CD25^{high} T cells, involving larger cohorts, in order to confirm and integrate our findings and obtain a deeper perspective on their role in CVID and AID.

Unquestionably, retrospective methodology has limited our clinical characterization and perception of AIDs evolution in this group, therefore demanding further follow-up studies with clinical and immunologic data simultaneous collection. Longitudinal studies will also be instrumental to clarify the prognostic value of CD4^{+}CD25^{high} allowing us to offer each patient optimal and individually adapted follow-up and treatment protocols.

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TABLES AND FIGURES

Table 1: Patients with autoimmune diseases - diagnosis, age at presentation and treatment.

Case Number	Gender/age	AID (age at presentation)	AID treatment
1	M / 44	AIHA (29);ITP (39); *alopecia areata* (30)	AIHA: OS; ITP: splenectomy, AZT, Cy; *alopecia areata*: topical steroids
2	F / 61	*Rheumathoid* Arthritis (40)	NSAIDs
3	F / 63	ITP (58)	
4	M / 42	Pernicious anemia (39)	Intramuscular vitamin B12
5	M / 71	Sjogren's Syndrome (60)	
6	M / 21	Crohn's disease (21); eritroblastopenia (20)	Cy + OS
7	M / 63	Pernicious anemia (60)	Oral cyanocobalamin
8	M / 34	Psoriasis (22)	
9	F / 54	Pernicious anemia (44)	Intramuscular vitamin B12
10	F / 45	Pernicious anemia (39)	Intramuscular vitamin B12
11	F / 44	Psoriasis (30)	*Topical treatment*
12	M / 40	*Alopecia areata* (29), hypothyroidism (7)	Topical steroids, Levothyroxine
13	F / 42	ITP (13), Crohn's disease (38)	ITP: OS, splenectomy. CD: OS, mesalazine, Cy
14	F / 30	*Rheumathoid* Arthritis (30)	OS, NSAIDs
15	F / 16	Evans´s Syndrome (AIHA, ITP) (10)	OS
16	M / 58	ITP (30)	OS
17	F / 25	Vitiligo; autoimmune hepatitis (9)	OS, AZT
18	M / 53	*Alopecia areata*, ITP (46)	OS
19	F / 55	*Rheumathoid* Arthritis (25)	OS, hydroxychloroquine

NOTE: **AIHA**, autoimmune hemolytic anemia; **AZT**, Azathioprine; **CD**, Crohn´s disease; **Cy**, cyclosporine; **F**, female; **ITP**, immune trombocytopenic purpura; **M**, male; **NSAIDs**, non-steroidal anti-inflammatory drugs; **OS**: oral steroids.

Table 2: Clinical profile of patients with AID

Case Number	Age at 1st symptoms / type	Age at diagnosis / beginning IVIG	Respiratory infections	Bronchiectasis	Chronic diarrhea	LH	Granulomas	Other clinical manifestations	IVIG (mg/Kg/month)
1	17 / RI	32 / 34	U+L	Yes	Giardiasis + NLH + intestinal amyloidosis A	Yes*	No	CMV adenitis	1360
2	7 / RI	58 / 58	U+L	Yes	Non- infectious	No	No	Frequent Herpes I infections, sepsis	513
3	40 / RI	58 / 59	U+L	Yes	Non- infectious	No	No	Frequent Herpes zoster infections (5x)	450
4	14 / RI	34 / 34	U+L	Yes	Infectious Non-infectious	Yes	Yes		640
5	51 / RI	58 / 59	U+L	Yes	Non- infectious / NLH	Yes	Yes		453
6	4 / RI	13 / 14	U+L	Yes	Infectious Non-infectious	Yes	No	Frequent Herpes I infections	667
7	25 / RI	57/ 59	U+L	Yes	Non- infectious / NLH	Yes	No	Acute colangitis - *E coli*, duodenal papilla cyst	594
8	6 / RI	24 / 24	U+L	Yes	Giardiasis Non-infectious	Yes	No		373
9	7 / RI	32 / 40	U+L	Yes	No	Yes	n.a.		n.a.
10	34 / RI	39 / 39	U+L	Yes	Infectious Non-infectious	No	No		784
11	25 / RI	41 / 41	U+L	Yes	No	No	No	Ferropenic anemia, sepsis (Klebsiella), duodenal ulcer	520
12	7 / AID	36 / 37	U+L	Yes	Infectious Non-infectious	Yes	No	Chronic prostatitis, hepatitis	480
13	13 / AID	30 / 30	U+L	Yes	Infectious Non-infectious	Yes*	Yes	Sepsis (*Morganella morganii*), frequent conjunctivitis, intestinal CMV	1820
14	30 / AID	49 / 50	U+L	Yes	No	Yes	No		380
15	10 / AID	12 / 12	L	Yes	Non-infectious / NLH	Yes	n.a.		n.a.
16	30 / AID	54 / 54	No	No	No	No	n.a.		n.a.
17	9 / AID	14 / 14	No	No	No	No	No		625
18	46 / AID	52 / 52	No	No	No	yes	n.a.		n.a.
19	25 / AID	36 / 36	U+L	Yes	Infectious diarrhea	No	No	Frequent cutaneous mycosis	373

NOTE: **AID**, Autoimmune diseases; **CMV**, Cytomegalovirus; **IVIG** intravenous immunoglobulin; **L**, lower respiratory tract; **LH**, lymphoid hyperplasia; **NLH**, nodular lymphoid hyperplasia; **RI**, respiratory infections; **U**, upper respiratory tract; **n.a.**; data not available; ***** status post splenectomy

Table 3: Immunological profile of patients with AID

Case number	Immunoglobulin levels at diagnosis						Cross-sectional evaluation													
	IgG	IgA	IgM	IgG1	IgG2	IgG3	Last IgG	WBC	Lym	CD3	CD4	CD4 counts	CD4/CD8	CD8	CD19	NK	HLA-DR+ within CD4	HLA-DR+ within CD8	Hb	Platelets
	mg/dL	Mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	cells /μL	cells /μL	%	%	cells /μL		%	%	%	%	%	g/dL	cells /μL
1	500	45	116	560	116	90	538	7500	3100	70	33	1023	1.2	28	17	11	19	43	13	103
2	466	30	50	419	59	21	894	5100	900	74	55	495	2.9	19	19	6	7	17	12	181
3	217	<7	9	209	23	5	992	3300	800	70	37	296	1.3	28	21	8	20	28	12,4	77
4	160	<7	17	168	11	<5	863	5800	1500	85	43	645	1.1	38	5	9	20	51	14.3	199
5	574	10	33	480	8	80	n.a.	6100	1300	n.a.	26	338	n.a.	n.a.	8	13	n.a.	n.a.	n.a.	n.a.
6	567	6	10	n.a.	n.a.	n.a.	604	7400	1400	77	19	266	0.4	49	3	18	n.a.	n.a.	12.3	227
7	174	<7	6	110	38	<5	451	10000	1800	n.a.	50	900	n.a.	n.a.	5.8	7	n.a.	n.a.	n.a.	n.a.
8	320	7	6	200	120	24	687	7700	1600	89	48	768	1.3	37	4	5	3	9	14.5	208
9	98	84	39	n.a.	n.a.	n.a.	591	3270	1242	82	35	434.7	0.8	45	7	10	36	81	13.0	103
10	389	<7	90	151	96	<5	413	9100	2000	76	44	880	1.5	29	11	10	4	6	8.9	509
11	445	71	191	381	10	22	903	6400	1600	71	43	688	1.8	24	11	16	3	5	12.3	292
12	549	<7	56	413	33	162	846	5500	1000	66	45	450	2.4	19	12	20	10	25	13.5	209
13	313	<10	42	200	60	8	578	11200	4100	61	21	861	0.5	39	3	33	45	71	8.3	547
14	385	<10	54	252	37	8	972	4700	900	71	31	279	0.8	38	15	13	16	32	13	260
15	505	35	62	288	52	95	660	11400	3300	82	64	2112	3.6	18	14	3	17	11	14.3	327
16	300	25	25	n.a.	n.a.	n.a.	542	5650	1510	80	48	724.8	1.5	32	8	12	37	52	15	186
17	438	<7	64	348	<5	12	610	9300	2900	80	39	1131	1.0	38	15	4	12	14	13.2	186
18	374	30	35	281	83	5	687	4120	1210	72	49	592.9	2.0	24	14	7	21	33	15	145
19	440	11	15	n.a.	n.a.	n.a.	913	2700	1000	NA	42	420	0.8	51	2	3	18	44	12.2	119

NOTE: **n.a.**; data not available

Table 4: Comparison of clinical and laboratory data between patients with and without AID

	All patients (n=47)	AID (n=19)	No AID (n=28)	AID vs. no AID (p value)
Gender (M/F)	25/22	9/10	16/12	> 0.05
Age (years)	**37.7 ± 16.0**	**46.5 ± 15.0**	**31.8 ± 14.0**	**0.0017**
Age at first symptoms (years)	15.6 ± 14.7	21,0 ± 14,4	13.5 ± 13.4	> 0.05
Age at diagnosis (years)	**28.4 ± 17.6**	**38.2 ± 16.0**	**22.7 ± 15.7**	**0.0022**
Age at beginning treatment (years)	**30.0 ± 16.7**	**39.0 ± 16.1**	**24.6 ± 15.4**	**0.004**
Time 1st symptoms – diagnosis (years)	**12.8 ± 12.4**	**17.1 ± 12.0**	**9.1 ± 9.7**	**0.022**
Time 1st symptoms – treatment (years)	**14.3 ± 12.7**	**18.0 ± 12.6**	**11.1 ± 9.9**	**0.05**
Bronchiectasis (n)	43 (91.5%)	16 (84.2%)	27 (96.4%)	> 0.05
Chronic non infectious diarrhea (n)	**19 (40.4%)**	**12 (63.15%)**	**7 (33.3%)**	**0.015**
Chronic infectious diarrhea (n)	22 (46.8%)	9 (47.4%)	13 (46.4%)	> 0.05
Splenomegaly (n)	14 / 45 (31.1%)	8 / 17 (47.1%)	6 (21.4%)	> 0.05
Lymphoid hyperplasia (n)	**21 (44.7%)**	**12 (63.2%)**	**9 (32.2%)**	**0.043**
Granulomatous disease (n)	3 (6.4%)	3 (15.8%)	0 (0%)	> 0.05
IVIG doses (mg/kg/month)	618.2 ± 511.3	668.9 ± 401.7	586.5 ± 575.4	> 0.05
IgG at diagnosis (mg/dL)	**294.5 ± 147.3**	**379.7 ± 141.7**	**234.5 ± 120.8**	**0.0009**
IgA at diagnosis (mg/dL)	22.5 ± 29.8	24.2 ± 26.8	21.3 ± 32.3	> 0.05
IgM at diagnosis (mg/dL)	43.8 ± 52.4	50.7 ± 46.5	38.8 ± 56.6	> 0.05
IgG1 at diagnosis (mg/dL)	**227.2 ± 126.1**	**297.3 ± 131.6**	**181.4 ± 100.8**	**0.0078**
IgG2 at diagnosis (mg/dL)	50.3 ± 54.9	50.1 ± 38.6	50.5 ± 64.2	> 0.05
IgG3 at diagnosis (mg/dL)	26.9 ± 34.2	36.5 ± 47.6	20.4 ± 19.6	> 0.05
Last IgG (mg/dL)	658.1 ± 147.4	695.9 ± 181.5	633.3 ± 117.4	> 0.05
Leucocytes (cells /μL)	6740 ± 2514	6644 ± 2626	6804 ± 2481	> 0.05
Lymphocytes (cells /μL)	1954 ± 1304	1745 ± 932.3	2096 ± 1505	> 0.05
CD3 (%)	76.3 ± 7.4	75.4 ± 7.4	76.8 ± 7.4	> 0.05
CD3 counts (cells /μL)	1529 ± 1091	1360 ± 700,9	1626 ± 1263	> 0.05
CD4 (%)	41.0 ± 11.4	40.6 ± 11.4	41.3 ± 11.1	> 0.05
CD4 counts (cells /μL)	759.3 ± 392.8	700.2 ± 429.2	799.4 ± 368.7	> 0.05
CD4/CD8	1.5 ± 0.8	1.5 ± 0.8	1.5 ± 0.8	> 0.05
CD8 (%)	32.5 ± 11.7	32.7 ± 10.3	32.4 ± 12.6	> 0.05
CD19 (%)	11.0 ± 4.9	10.2 ± 5.8	11.5 ± 4.3	> 0.05
CD19 counts (cells /μL)	217.6 ± 175.7	174.9 ± 143.0	246.5 ± 191.9	> 0.05
NK (%)	10.1 ± 6.5	11.0 ± 7.2	9.5 ± 6.0	> 0.05
HLA-DR + cells within CD4+ (%)	16.7 ± 12.8	18.0±12.4	15.7±13.3	> 0.05
HLA-DR + cells within CD8+ (%)	28.1 ± 19.6	32.6 ± 23.0	24.7 ± 16.2	> 0.05
Hb (g/dL)	13.1 ± 1.7	12.8 ± 1.8	13.4 ± 1.6	> 0.05
Platelets (cells /μL)	219.0 ± 91.7	228.1 ± 131.3	213.3 ± 56.6	> 0.05

NOTE: 1 - Data are presented as mean ± SD, unless indicated otherwise; 2 - IgA level was below the cut-off of the test in 10/19 patients; cut-off value (7mg/dL) was therefore used to calculate the mean and SD. **IVIG**, intravenous immunoglobulin.

Table 5: CD4^{+}CD25^{+} quantification

	All patients CVID (n=47)	AID (n=19)	No AID (n=28)	Controls (n=29)
Gender (M/F)	25 / 22	9 / 10	16 / 12	13 / 16
Age	37.7 ± 16.0	**46.5 ± 15.0** ††	**31.8 ± 14.0** $^{\S\S\ **}$	**40.8 ± 9.5** ††
CD4^{+}CD25^{+}	16.8 ± 6.7	**14.2 ± 4.4** $^{*\ \dagger}$	**18.4 ± 7.5** §	**17.1 ± 4.8** §
CD4^{+}CD25^{high}	**0.96 ± 0.63** *	**0.65 ± 0.37** $^{***\ \dagger\dagger}$	**1.17 ± 0.69** §§	**1.25 ± 0.36** $^{\#\ \S\S\S}$
CD4^{+}CD25^{high}RO^{+}	96.1 ± 4.3	96.6 ± 5.4	95.8 ± 3.4	96.52 ± 2.75

NOTE: Data are presented as mean ± SD

CD4^{+} CD25^{+} - percentage of CD25^{+} cells within CD4^{+} T cells

CD4^{+} CD25^{high} - percentage of CD25^{high} within CD4^{+} T cells

CD4^{+} CD25^{high}RO^{+} - percentage of CD45RO^{+} cells within CD4^{+} CD25^{high} T cells

$^{\#}$ Significance in comparison with all patients with CVID: $^{\#\#\#}$ p<0.001; $^{\#\#}$ p<0.01; $^{\#}$ p<0.05

§ Significance in comparison with patients with AID: §§§ p<0.001; §§ p<0.01; § p<0.05

† Significance in comparison with patients with no AID: ††† p<0.001; †† p<0.01; † p<0.05

* Significance in comparison with controls: *** p<0.001; ** p<0.01; * p<0.05

Figure 1

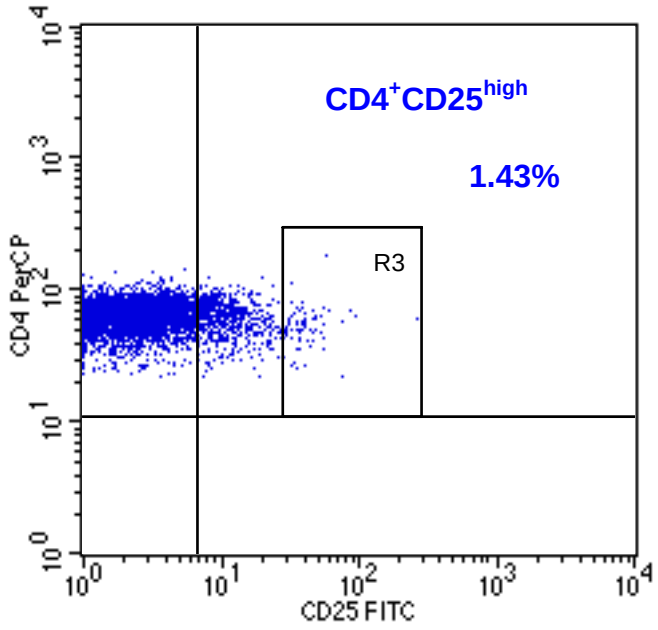


Figure 1 – **Flow cytometry**

$CD4^+CD25^{high}$ were quantified in whole blood on a Fluorescense Activated Cell Sorter FACSCalibur (Becton-Dickinson®). We show dot plot of a healthy control illustrating $CD4^+CD25^{high}$ definition adopted in this work based on Baecher-Allan *et al* [34], in which $CD4^+CD25^{high}$ cells appear as *a tail to the right from the major population containing both* $CD4^+CD25^{low}$ *and* $CD4^+CD25^-$ *cells*.

Figure 2

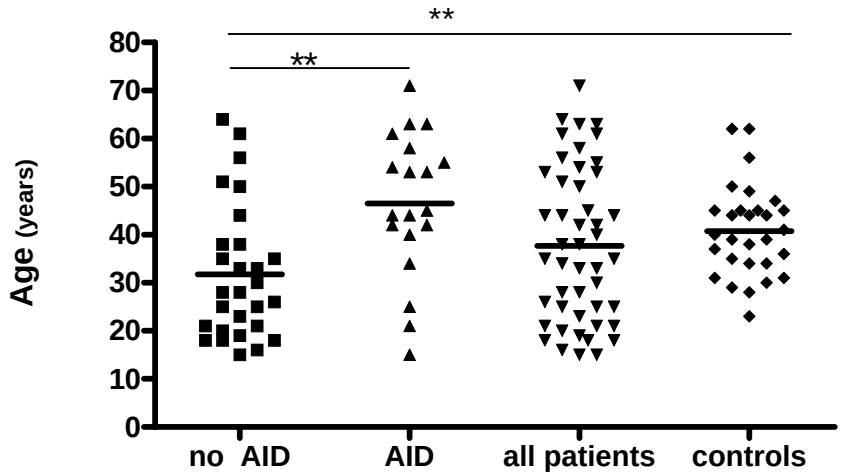

**
**
80
70
60
50
40
30
20
10
0
Age (years)
no AID
AID
all patients
controls

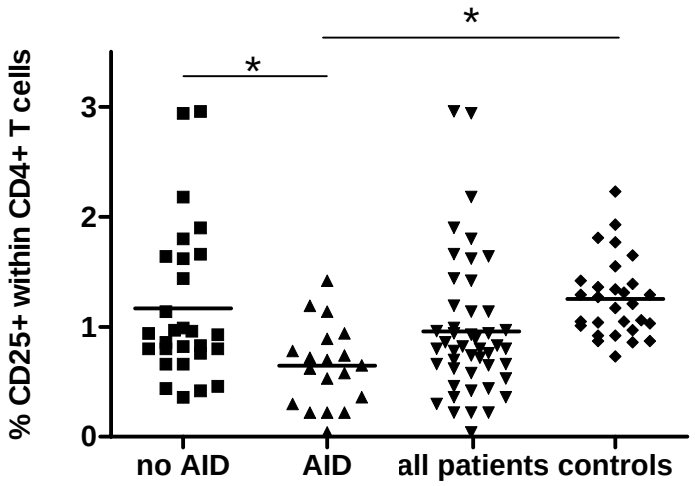
Figure 2 - **Age distribution of the cohorts**

Age distribution of healthy controls and CVID patients with and without AID. Each dot represents one individual. Bars represent means. Statistical significance between groups:

* $p<0.05$; ** $p<0.01$; *** $p< 0.001$.

Figure 3

A


*
*
% CD25+ within CD4+ T cells
3
2
1
0
no AID
AID
all patients
controls

B

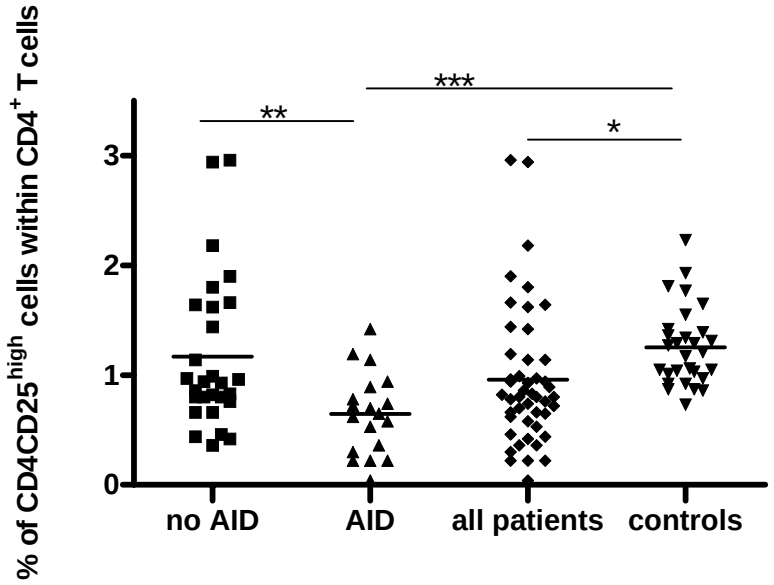

***
**
*
% of CD4CD25high cells within CD4+ T cells
3
2
1
0
no AID
AID
all patients
controls

Figure 3 – **Analysis of the expression of CD25 within $CD4^+$ T cells**.

Patients with CVID and healthy controls were evaluated for CD25 expression within $CD4^+$ T cells by flow cytometric analysis of the intensity of fluorescence: (A) Proportion of $CD4^+$ T cells that express CD25 and (B) Proportion of $CD4^+$ T cells that express high intensity of CD25 fluorescence. For comparison between patients with and without AID, results are shown in different columns. Each dot represents one individual. Bars represent means. Statistical significance between groups: * $p<0.05$; ** $p<0.01$; *** $p< 0.001$.

Figure 4

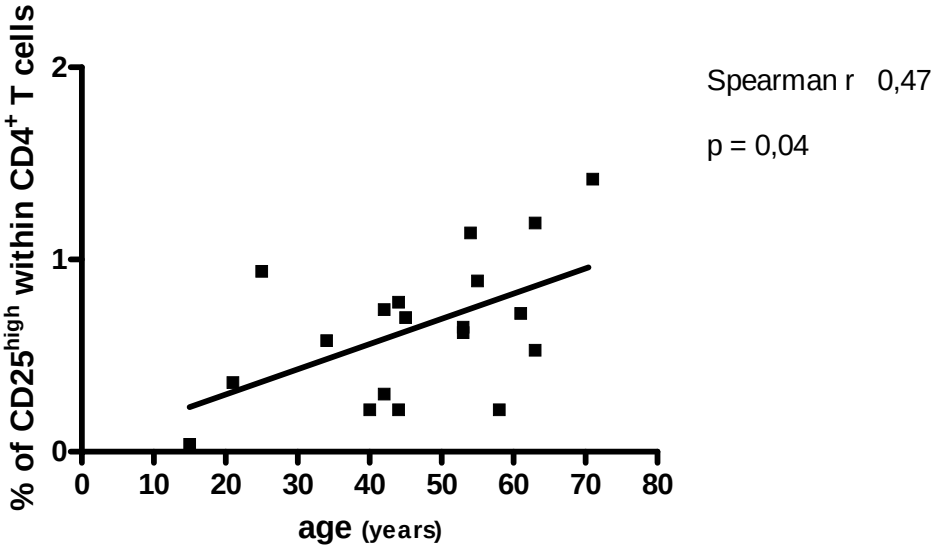

% of CD25high within CD4+ T cells
2
1
0
0
10
20
30
40
50
60
70
80
age (years)
Spearman r 0,47
p = 0,04

Figure 4 – **Analysis of the correlation between age and $CD4^+CD25^{high}$ in patients with CVID and AID**.

Graph illustrating the positive correlation between age and proportion of $CD4^+CD25^{high}$ within $CD4^+$ T cells. No significant correlation was found between these two variables when analyzing controls or patients without AID.

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The specific contributions of each co-author to this manuscript are indicated bellow:

Susana Lopes da Silva*
Designed research
Collected all clinical data
Analysed data
Discussed the results
Wrote the paper

Drahomira Detkova‡
Designed research
Performed flow cytometric analysis
Performed and analyzed all CD4+CD25+ analysis
Analysed data
Discussed the results

Antonio Alvarez§**, Javier de Gracia**§**, Jose Maria Bertran**†
Clinical collaboration

Manolo Hernandez‡
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